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NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

ANNUAL REPORTS

DIVISION OF INTRAMURAL RESEARCH

October 1, 1990 to September 30, 1991

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91

TABLE OF CONTENTS

PREFACE

Dr. Phillip Gorden, Director I

DIVISION OF INTRAMURAL RESEARCH

Dr. Allen Spiegel, Director
Dr. Edward Steers, Deputy Director

PROJECT REPORTS

MATHEMATICAL RESEARCH BRANCH

Summary 1
Mathematical formulations and analysis relevant to experimental neurophysiology 9
Mathematical description of substrate transport in capillary-tissue structures 10
Mathematical description of cellular neuroelectric signal transmission 11
Electrical and chemical oscillations in coupled cell systems 12

LABORATORY OF CELLULAR AND DEVELOPMENT BIOLOGY

Summary 13
Protein nucleic acid interactions: chromatin structure and function 22
Study of ribonuclease and its inhibitor from bacillus amyloliquefaciens 23
Studies of folic acid (dihydrofolate reductase) 24
Synthesis and transport of lipoprotein and hepatic lipases in tissues and cells 25
Ultrastructural immunocytochemistry of lipid metabolism in cultured cells and tissues 26
Large-scale processing of biological material 27
Regulation of developmental gene expression 28
Regulation of adipocyte metabolism 29
Control of gene expression in early mammalian development 30
Chromatin structure in regulation of mammalian gene expression 31

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Summary 32
The role of the carbohydrate moiety of glycoprotein in cellular activity 39
Enzymatic basis of detoxication 40
Polysaccharides in morphogenesis 41
Thermodynamic and kinetic studies of protein structure and enzymic mechanisms 42
The role of the nuclear envelope in intracellular protein sorting 43
Tissue specific and hormone regulated gene expression 44
Inherited Disorders of Lysosomal Function 45
Electrochemical ion gradients as a mechanism of cellular message transmission 46
Cell regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes 47
Endocytosis, secretion and compartmentalization in mutant CHO cells 48
The role of intracellular traffic in HIV infection 49
Cell specific activity of elements within the HIV-LTR 50
Direct measurement of forces between membranes or macromolecules 51
Physics of ionic channels and other proteins with aqueous cavities 52
Structure and physical properties of DNA and DNA - protein complexes 53
Histamine release on a hydration of Granule matrices 54
Cell-cell fusion due to influenza hemagglutinin 55

LABORATORY OF CHEMISTRY

Summary 56
Reactions and immunochemistry of carbohydrates 57
Analogues of thyrotropin - releasing hormone 58
Stereopopulation control in drug delivery and enzyme simulation 59
Chemistry of bioimidazoles 60
Halogenated biogenic amines in biochemistry and pharmacology 61
Significance of ligand tautomerism in biorecognition 62
Functionalized cogeners of bioactive compounds 63
Prosthetic groups for radiolabeling of functionalized drugs and peptides 64
Development of drugs acting at adenosine receptors 65
Aldose reductase inhibitors 66
Novel amino acids for conformational and stereochemical constraints in peptides 67
Florinated analogue of bioactive peptides 68

LABORATORY OF CELL BIOLOGY AND GENETICS

Summary 69
Cytogenetics 74
Mechanisms of hormone and transmitter secretion 75

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

Summary 76
Biochemistry of sulfur-containing compounds 88
Aldoheptose biosynthesis and its regulation and hepatitis non-A, non-B 90
Mammalian transposons 91
Bacteriophage T4 gene expression 92
Structure and function of the tryptophan synthase multienzyme complex 93
Noncovalent: intermolecular interactions in biochemistry 94
Enzymatic mechanisms of DNA replication: The bacteriophage T4 system 95
Structure and interactions of biologically important macromolecules 96
Polyamine biosynthesis and function 97
Yeast RNA virology 98

LABORATORY OF CHEMICAL BIOLOGY

Summary 99
The core loop interaction that controls protein folding 101
Studies of protein folding problems.......... 102
Trans-acting factor(s) controlling globin gene expression in K562 cells 103
Sickle cell anemia: The intracellular polymerization of hbS 104
The mechanism of antigen-antibody interaction 105
The development of non-invasive methods to assess sickle cell patients 106
Regulation of beta globin gene expression 107
Laboratory model of adult globin gene expression 108
Trans-activating factors and globin gene expression: A direct approach 109

The Epsilon Gene Silencer: Characterization of Trans-acting Factor 110
Human Erythopoietin Receptor Gene: Cloning and Transcription Analysis 111
Effect of hydroxyurea on fetal hemoglobin synthesis in Congenital Hemoglobin Disorders.......... 112
Cytogenetic investigations of patients with genetically determined disorders 113

Aids: transcriptional regulation by the TAT-protein LTR of HIV in vitro ... 114
Coordinated expression of human beta sickle antilles and human alpha globin in transgenic mice ... 115
Analysis of the Epsilon Globin gene flanking sequences ... 116
Trans-regulation of human globin genes ... 117
The erythropoietin receptor and genetic control red cell development ... 118
Mechanism(s) of enhanced gamma globin gene expression in patients ... 119
Production and Characterization of HIVTAT ... 120
Characterization of Transcription Factors Binding to the Epsilon Globin Silencer ... 121
Beta-Globin gene expression in patients with different type of βeta-thalassemia mutation ... 122
Globin Expression in an Erythroid Progenitor Culture System ... 123

LABORATORY OF CHEMICAL PHYSICS

Summary ... 124
Molecular dynamics and vibrational characteristics of membrane assemblies ... 128
Asymmetric synthesis: Structure, stereochemistry, and NMR ... 129
The structure and dynamic properties of macromolecules ... 130
Structure and interaction of biomolecules ... 131
Electric and molecular structural investigation ... 132
Dynamics of proteins and studies on sickle cell disease ... 133
The physics and chemistry of photoreception ... 134
Macromolecular dynamics and assembly reactions ... 135
Spectroscopic investigation of membrane lipids and models ... 136
Theoretical studies on the dynamic aspects of macromolecular function ... 137
Nuclear magnetic resonance: New methods and molecular structure determination ... 138
Conformation and dynamics of biological macromolecules ... 139
Structural studies of AIDS proteins by NMR ... 140
Determination of three-dimensional structures of macromolecules in solution by NMR ... 141
Investigations of macromolecular structures and dynamics solution by NMR ... 142
NMR and other spectroscopic studies of molecular structure ... 143
Theoretical studies of dynamical processes in chemical physics and biophysics ... 144

Free Energy Conversion in Biology 145

LABORATORY OF BIOORGANIC CHEMISTRY

Summary 146
Pharmacologically active compounds from amphibians and other natural sources 189
Pharmacology and metabolism of biogenic amines and related compounds 190
Ion channels receptors and second messengers in the nervous system 191
Enzymatic oxidation of drugs to toxic and carcinogenic metabolites 192
Mechanistic enzymology of HIV proteins 193
Mass spectrometry of drugs, metabolites and natural products 194
Adenosine receptor agonists and antagonists 195
Interaction between second messengers systems 196
Analogues of Thyrotropin-releasing Hormone 197
Stereopopulation Control in Drug Delivery and Enzyme Simulation 198
Chemistry of Bioimidazoles 199
Halogenated Biogenic Amines in Biochemistry and Pharmacology 200
Significance of Ligand Tautomerism in Biorecognition 201
Functionalized Congeners of Bioactive Compounds 202
Prosthetic Groups of Radiolabeling of Functionalized Drugs and Peptides 203
Development of Drugs Acting at Adenosine Receptors 204
Aldose Reductase Inhibitors 205
Novel Amino Acids for Confromational and Sterochemical Constraints in Peptides.................. 206
Fluorinated Analogues of Bioactive Peptides 207
Chemistry and Biology of Novel Pyrimidine and Purine Necleosides 208
Antimalarial Agents Based on Bioheterocycles 209

LABORATORY OF MOLECULAR BIOLOGY

Summary 210
Studies of functions involved in genetic recombination 220
Studies of immunoglobulin gene rearrangement 221
Studies on mechanism of genetic recombination 222
Chromatin structure and function 223
Enzyme structure 224
Three-dimensional structure of proteins of the immune system 225
Chemical and structural investigations of nucleic acids and related molecules 226
Replication, recombination, and repair of microbial DNA 227

Nonheritable antibiotic resistance 228
Mammalian DNA replication, regulation and amplification 229
Energy conversion in biology 230
Statistical thermodynamics of protein and polynucleotide systems 231

Thermal measurements of biomolecular systems 232
Influences of macromolecular crowding on biochemical systems 233
Developmental regulation of differential gene expression 234
Studies on the mechanism of retrovial DNA integration 235
AIDS related proteins: Structure and function 236
Control of gene expression during chicken erythrocyte development 237
Structural molecular biology 238
Regulation of a gene expressed in undifferentiated teratocarcinoma cells 239
Channeling in the biosynthesis of histidine 240
Structural Studies of Molecular Recognition 241
Study of the Potential Use of Catalytic Antibodies Against AIDS 242

METABOLIC DISEASES BRANCH

Summary 243
Structure, secretion, and mechanism of action of parathyroid hormone 252
Studies on the mode of action of thyrocalcitonin 253
Study of hyperparathyroidism: Etiology, diagnosis, and treatment 254
Vitamin D resistance and related disorders 255
Regulation of mineral metabolism 256
Disorders of immune regulation in patients with systemic lupus erythematosus 257
Production and characterization of nephritic factor 258
Regulation of human immune response by complement 259
Immunosuppressive drug therapy in lupus glomerulonephritis 260
Renal biopsy pathology in systemic lupus erythematosus 261
Glomerular Disease Transgenic Mice 262
Morphometry of renal lesions in Pima Indians 263
Studies of Glomerular Cells Derived from Transgenic Mice.... 264
Renal lesions in leukemias, lymphomas, and carcinomas 265
Biology of insulin receptors in glomerular cells 266
Pathogenesis of murine lupus nephritis 267
Membranes lupus nephropathy 268
Glomerular change due to GH and IGF-I 269
Role of IGF-I in biology of mouse glomerular cells 270

Biology of human glomerular mesangial cells 271

Proteoglycan production by mouse glomerular epithelial cells 272
Idiopathic membranous nephropathy 273
Renal lesions in the ablation model: Role of growth factors 274
Identification of IGF-I binding proteins by mouse mesangial cells 275

Interactions between TGF-B and glomeruli 276
Glomerular lesions in non-obese diabetic mice 277
Advanced Glycosylation End Products, Effect on Mesangial Cells 278

Production of Metalloproteinases and TIMP1 by Glomerular Cells 279
Gene Expression in Microdissected Mouse Glomeruli 280
Effects of glucocorticoids on T cell activation 281
Transcriptional regulation of immunoglobulin genes.......... 282

CLINICAL ENDOCRINOLOGY BRANCH

Summary 283
Thyroxine-protein interactions 287
Structure of polypeptide and protein hormones 288
Studies in thyroid diseases 289
Membranes and secretion 290
Thyroid hormone secretion and the function of microtubules 291
Adenylate cyclase and other extracellular products of B pertussis 292
Synthesis of Throxine Transport Proteins 293
Thyroid hormones - cell interactions 294
Mapping of triiodothyronine responsive genes 295
Regulation of specific rat liver mRNAs by thyroid hormone 296
Molecular biology of thyroid hormone receptors 297
Effect of thyroid hormone on synthesis of myelin basic proteins 298
The role of POU - domain genes during Xenopus laevis embryogenesis 299
The role of XPO, a localized zinc finger gene, in anterior-posterior axis formation in xenopus 300

DIABETES BRANCH

Summary 301
Phosphorylation of the insulin and IGF-I receptor 308
Insulin gene expression and insulin action 309
Studies of insulin receptors in circulation cells in man 310

Antibodies to receptors: Detection in disease states and use as probes 311
Positron emission tomography 312
Acromegaly and growth hormone 313
Cellular hormone-like peptides 314
Morphologic studies of ligand binding to cells 315
Insulin receptors in syndromes of extreme insulin resistance 316
Biosynthetic labeling of the insulin receptor 317
Tissue receptors for insulin and insulin-like growth factors 318
Tyrosine-specific protein kinase activity associated with the insulin receptor 319
Use of SMS 201-995 in hormone secreting tumors 320
Insulin Gene Expression and Insulin Action 321

CLINICAL HEMATOLOGY BRANCH

Summary 322
Study of immunology of blood cell deficiencies 328
Study of blood coagulation and diseases of hemorrhage and thrombosis 329

GENETICS AND BIOCHEMISTRY BRANCH

Summary 330
Gene expression and human genetics 333
Toxins and DNA repair in xenopus oocytes 334
Structure-function relationship of lysosomal enzymes 335
CD4 receptor structure/function project 336
Molecular studies of protein-DNA interactions 337

DIGESTIVE DISEASES BRANCH

Summary 338
Studies of membrane function 348
Gastrointestinal hormones 349
Cyclic nucleotide mediated functions 350
Identification and characterization of Receptors for GI Peptides 351
Cellular basis of action of gastrointestinal peptides 352
Management of Islet cell tumors 353
Receptors on gastric smooth muscle cells 354
Studies relating to the pathogenesis of hepatic encephalopathy 355
Immunologic studies of primary biliary cirrhosis 356
Studies of the natural history and treatment of chronic type B hepatitis 357
Studies of natural history and treatment of chronic non-A, non-B, (type C) hepatitis 358
Trials of therapies for primary biliary cirrhosis 359
Immunological studies in chronic viral hepatitis 360

Studies of the opiate system in cholestatic liver disease 361

MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH

Summary 362
Biosynthesis and glycosylation of thyrotropin 369
Molecular biology of pituitary glycoprotein hormones and hypothalamic releasing hormones 370
Insulin-like growth factors 371
Insulin-cell interaction 372
Insulin's regulation of glucose transport 373
Alterations in insulin's action in insulin-dependent diabetes mellitus 374
Insulin's regulation of hormone binding 375
Counterregulation of insulin's action by catecholamines 376
Alterations in insulin's action with fasting/refeeding 377
Mutations of the thyroid hormone receptor gene in patients with thyroid hormone resistance 378

LABORATORY OF STRUCTURAL BIOLOGY

Summary 379
Biology of complex carbohydrates 381
Metabolism and role of polysaccharide sulfate 382
Expression and function of bacterial cell surface components in pathogenesis 383

LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY

Summary 384
Function of DNA virus genomes in animal cells 389
Hormonal regulation of cell growth and differentiation 390
Studies on the metabolic defect in Sialuria 391
Regulation of HIV by AAV 392
Initial Intracellular Events of Steroid Hormone Action...... 393

LABORATORY OF ANALYTICAL CHEMISTRY

Summary 394
Analytical services and methodology 400
Initial intracellular events of steroid hormone action 401
The development of methods and materials for the study of medical problems 402
Professional practices of biomedical scientists 403
Interferon induction and action. The antiviral activity of nucleoside analogs 404

Physostigmine and analogs 405
Mammalian alkaloids 406
Structure-Activity relationships of colchicinoids based on tubulin binding 407
Analogues of nucleic acids and their components as potential anti-AIDS agents 408
Nortropane alkaloids 409
Analytical reagents from dihydrofluorescein 410

LABORATORY OF NEUROSCIENCE

Summary 411
Receptors for neurotransmitters and drugs in brain and peripheral tissues 413

MOLECULAR PATHOPHYSIOLOGY BRANCH

Summary 414
Molecular biologic studies on the cause of parathyroid neoplasia 418
Guanine nucleotide binding proteins as receptor-effector couplers 419
Studies on pseudohypoparathyroidism and related disorders 420
Studies on McCune-Albright Syndrome................................ 421

LABORATORY OF MEDICINAL CHEMISTRY

Summary 422
Design and synthesis of drugs acting on central and peripheral tissues 445
Design, synthesis and evaluation of medicinal agents and research tools 446
Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agent 447
Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs................................ 448
Reactions and Immunochemistry of Carbohydrates................................ 449

PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH

Summary 450
Diabetes mellitus and other chronic diseases in the Gila River indian community 461
Complications and outcome of diabetic and prediabetic pregnancies 462
Gila River indian community autopsy and mortality study 463
Natural history of arthritis and rheumatism in the Gila River Indian Community 464
Cross-sectional and longitudinal study of "prediabetes" in the Pima Indians 465

Insulin resistance and the regulation of muscle glycogen synthase activity 466
Energy expenditures in Pima Indians: risk factors for body weight gain 467
WHO collaborating center for epidemiological and clinical investigations in Diabetes 468
Treatment of impaired glucose tolerance in Malmohus County, Sweden 469
Role of insulin receptor tyrosine kinase in insulin resistance in Pima Indians 470
Genetics of non-insulin dependent diabetes mellitus 471
Regulation of skeletal muscle ribosomal protein S6 kinase by insulin 472
Contribution of protein tyrosine phosphatase to insulin resistance 473
Regulation of physhorylase phosphatase by insulin 474
Regulation of skeletal muscle casein kinase II by insulin 475
Relationship between insulin resistance and blood pressure 476
The long Q-T interval syndrome in diabetes mellitus 477
Autonomic nervous system activity in obesity 478
Epidemiology of complications of non-insulin-dependent diabetes 479
Kidney function in non-insulin-dependent diabetes mellitus 480
Insulin and hypertension in Pima Indians 481
Dietary survey of the Pima Indians of the Gila River Indian Community 482
Sodium - lithium countertransport and blood pressure 483
Insulin resistance in obesity and the association with lymph insulin kinetics 484
Effect of nicotinic acid-induced insulin resistance on β-cell function 485
Regulation of gene expression by insulin 486
Insulin resistance in obesity and the associations with membrane phospholipid................................. 487

XII

PROJECT NUMBERS

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

ACTIVE PROJECTS

Z01 DK 13001-18 MRB
Z01 DK 13002-19 MRB
Z01 DK 13004-18 MRB
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Z01 DK 29001-19 LCP
Z01 DK 29005-17 LCP
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Z01 DK 29007-20 LCP
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Z01 DK 29010-19 LCP
Z01 DK 29011-20 LCP
Z01 DK 29016-15 LCP
Z01 DK 29017-12 LCP
Z01 DK 29019-11 LCP
Z01 DK 29020-07 LCP
Z01 DK 29021-06 LCP
Z01 DK 29022-04 LCP
Z01 DK 29023-04 LCP
Z01 DK 29025-03 LCP
Z01 DK 29026-03 LCP
Z01 DK 29027-03 LCP
Z01 DK 29028-01 LCP
Z01 DK 31100-26 LBC
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Z01 DK 31107-04 LBC
Z01 DK 31108-03 LBC
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Z01 DK 31110-15 LBC
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INACTIVE PROJECTS

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TERMINATED PROJECTS

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Annual Report of the

Mathematical Research Branch

National Institute of Diabetes and Digestive and Kidney Diseases

Current research projects of the Mathematical Research Branch reflect a broad range of interests in the development and application of theoretical models and of quantitative methodologies for understanding biological systems.

This research involves several different collaborations within the Branch and with other research groups, both at the NIH and elsewhere. This report describes recent work in the areas of cellular and network neurobiology, electrical activity of secretory cells, microcirculation, renal physiology, egg fertilization, cell energetics, and mathematical/numerical methodologies.

During the past year, international collaborative projects have involved foreign investigators at Hebrew University, Jerusalem (Department of Neurobiology) and at the Universite Nacional Autonomous de Mexico, Mexico City (Department of Mathematics); MRB scientists participated in securing NSF Binational grants for these projects. Invited presentations were given by J Rinzel at distinquished symosia (Nonlinear Oscillations, Waterloo, Canada; Gordon Conference on Theoretical Biology; Systems of Coupled Oscillators, Seattle). An invited overview paper was completed by W Rall (The Neurosciences: Paths of Discovery). J Rinzel was invited to teach in the intensive course on Computational Neuronal Modeling (Marine Biological Laboratory, Woods Hole) and in a condensed version of the course in Mexico City. The MRB is sponsoring at the NIH a one-day satellite meeting, Theoretical Biology Day, to the Second International Conference on Industrial and Applied Mathematics, July 7, 1991. W Rall and J Rinzel were invited as extended-term visitors (2-4 weeks) to consult with students and faculty on computational neurobiology at UC-Berkeley and UC-Davis, respectively. MRB scientists serve on the Editorial Boards of seven scientific journals.

Network Neurobiology

Oscillations of a mutually inhibitory network. Previously we formulated a kinetic model of the T-type calcium current based on data from thalamic neurons. Our model cell with this current can exhibit postinhibitory rebound excitation, like the low-threshold spike (LTS) of thalamic neurons (with M Rogawski, NINDS). Now we consider a pair of such cells coupled by inhibitory synapses. Alternating oscillations occur naturally in such systems, and we analysed these using tools from the theory of nonlinear dynamics. This classical out-of-phase oscillation was discussed in the context of pacemakers for central pattern generators. Moreover, when the synaptic current has a slow decay property, perfect in-phase synchrony can be realized. Our results may apply to the 10 Hz synchronized bursting oscillations seen in the mammalian thalamocortical system. The thalamic reticular nucleus, which consists primarily of cells possessing T-type channels and interacting via GABAergic synapses, was proposed as a pacemaker. We are extending our analysis to a network of many cells. (X J Wang and J Rinzel)

Rhythmogenic effects of weak electrical coupling. In modeling electrical bursting activity of pancreatic beta-cells we found that coupling by gap junctions can significantly increase burst period of an ensemble. Period extension occurs when the coupling is weak: adequate to synchronize the bursts, but not the spikes within a burst. By using models of cell pairs, we find that spikes can phase-lock 180 degrees apart. When spiking out-of-phase, an excited cell depolarizes its neighbor, keeping it above threshold and enabling the pair to oscillate at parameter values for which the cells individually are silent. This alters the parameter range for which oscillations occur and leads to period extension when bursters are coupled. Out-of-phase spiking has other effects as well. For example, two non-bursting pacemaker cells when coupled may burst. Thus, electrotonic coupling is capable not only of synchronizing cells, but also of modulating electrical rhythms in more subtle ways. Our results apply to a large class of coupled cell oscillators. (A Sherman and J Rinzel)

Coupled oscillators near saddle loop bifurcations. For a class of neuron models, oscillations terminate as a parameter is varied due to a saddle loop bifurcation in which the oscillation period goes to infinity. We are studying two identical gap-junction coupled cells which are each near a saddle loop bifurcation. Preliminary calculations and numerical simulations suggest that for coupling strengths below a certain threshold, the oscillators generically are not stably in-phase near the saddle loop bifurcation. For weak coupling the oscillators are anti-phase, while for moderate coupling strengths other behaviors can occur. (T Kiemel and J Rinzel)

Activity patterns in slow synapse networks. In some neurons chemical synapse kinetics are much slower than spike generating processes. Exploiting this time scale difference, we apply the method of averaging to obtain a reduced model for the slow synaptic variables. Using phase plane analysis we understand bistable activity of two coupled inhibitory neurons, and we discover a novel mechanism for bursting of an excitatory-inhibitory cell pair. The latter behavior was captured only because our "averaging" was done on a biophysical spike-generating model, rather than on an overidealized model. Moreover, such bursting appears to be typically chaotic. Also, using perturbation methods we (T Kiemel and P Frankel) have described the phase-locking effects of slow coupling, and we find them fundamentally different than from weak coupling. This requires understanding coupled "cylinder-dynamics" instead of coupled "circle-dynamics" models. (P Frankel and J Rinzel)

The lamprey central pattern generator for swimming. The isolated spinal cord of the lamprey can produce a travelling wave of neural activity as evidenced by repetitive bursts of action potentials recorded at the motor roots. The time delays between bursts measured at different segments and the burst periods typically fluctuate about some mean. We are performing a time series analysis of these fluctuations. We have found high correlations between burst periods at different segments, indicating that the fluctuations at least partially reflect the underlying coordinating system. Autocorrelations between time delays measured a cycle or more apart are nearly zero, suggesting that the coupling between segments is strong. We are formulating a stochastic model of the system in order to interpret other aspects of the fluctuations. (T Kiemel and A Cohen, N Mellon: Univ MD)

Cellular Neurobiology

Analytical solutions for a dendritic neuron modeled as coupled cylinders. Specific examples were developed for illustrating the analytical solutions of several boundary value problems (BVP) that correspond to several types of simplified neurons. These models are composed of coupled cylinders, and each cylinder may represent an equivalent dendritic tree. Each BVP assumes passive (linear) membrane properties, with several cylinders coupled to a point soma; the far end of each cylinder is assumed "sealed". We consider first two cylinders of the same diameter, but having unequal lengths, then extend to a more general case of multiple cylinders having unequal diameters and lengths. In each case, expressions are given for the eigenvalues (and their corresponding time constants), and also the eigenfunctions, together with expressions for the coefficients required for any specification of the point location of input and the point of observation. Numerical examples illustrate that these solutions can be implemented simply with a hand calculator. Valuable insights are obtained by contrasting the roles of odd and even eigenfunctions and by focusing attention on the relative values of the coefficients. The resulting insights provide a basis for understanding the results of much more elaborate computations involving thousands of compartments and requiring matrix inversion on a super-computer. (W Rall)

Bifurcation analysis of thalamic neuron bursting. A burst of fast sodium spikes often rides the depolarization of a low-threshold spike (LTS) in thalamic neurons. A model, due to Hindmarsh and Rose, is qualitatively similar to ours for LTS generation (see above) but it also includes fast sodium spiking. The burst response is terminated by the slow inactivation of a low-threshold Ca++ current; this variable serves as the bifurcation paramater, in a fast-slow dissection, for understanding the sequence of burst, rest and tonic responses to different levels of background current. An analysis of the transition from one response to the other is being pursued. For example, with greater hyperpolarizing input the model's response changes from a relaxation-like oscillation, which is subthreshold for sodium spikes, to a characteristic repetitive burst pattern. (M Rush and J Rinzel)

Electrical Activity of Insulin-secreting Cells.

Ionic channel mechanisms for beta-cell bursting oscillations. Very slow voltage-gated inactivation of a calcium current was proposed to underlie the active-to-silent phase transition of a burst. Previously, we constructed a model based on experimentally determined HIT cell calcium channel parameters with this slow inactivation, and simulated bursts. Recently, experimental data has become available for calcium channels in the mouse beta-cell. We revised the model accordingly. The model no longer bursts, primarily because of a sharp lower threshold in the experimental calcium I-V relation. We conclude that slow voltage inactivation is unlikely to underlie bursting in the mouse pancreatic beta-cell unless the channel properties in the intact cell are considerably different from those in the "whole-cell" configuration used experimentally. However, a different model for bursting exists, based on periodic variation of the cytoplasmic ATP/ADP ratio. This model, when revised to reflect the recent calcium channel data, can still produce bursts that resemble experiment; it is our best candidate for explaining beta-cell bursting. We are collaborating with experimentalists to design studies for examining variations in the ATP/ADP ratio. (P Smolen and J E Keizer: UC-Davis)

Temperature dependence. Isolated beta-cells typically exhibit constant spiking (patch clamp recordings at room temperature) rather than bursting, as found in intact islets (microelectrodes at body temperature). These differences motivate several studies. The temperature dependence of bursting in the islet was investigated in further experiments (C Stokes with I Atwater, LCBG). We find that more than half of the islets, when cooled to 22-25 C, exhibited constant spiking, suggesting that temperature differences might account for the discrepancies. In addition, voltage-clamping a beta-cell within an islet was attempted to identify ionic channels which contribute to bursting under physiological conditions (body temperature) (C Stokes with E Rojas and I Atwater, LCBG). We are providing a theoretical framework for analyzing these data, which requires separating native membrane currents in the clamped cell from coupling currents flowing between neighboring cells. We have begun computations using our previous whole islet model (125 cells coupled by gap junctions) and using a simpler three-cell model (the clamped cell, a representative nearest neighbor, and a representative distant cell). Both models exhibit damped oscillations similar to those observed experimentally in low glucose with the clamped cell significantly depolarized. Our results suggest that the oscillations are due to coupling current from the near neighbors, rather than native channels. (C Stokes, A Sherman and J Rinzel)

Ionic coupling of beta-cells. We have continued to investigate the role of extracellular K+ in coupling the electrical bursting oscillations of an islet. Our model islet (with equations for bursting in each cell and with a reaction-diffusion equation for the K+ distribution in the interstitial spaces) exhibits synchronous bursting, as does a real islet in vitro. We are further exploring parameter dependencies and the mechanism of synchronization (using a more idealized model). Related to this modelling work, we have used brief K+ pulses to study experimentally phase-resetting of a bursting islet (C Stokes with I Atwater, LCBG). With rapid pulsing we could entrain the islet to a higher than intrinsic frequency. These results support the theoretical findings that K+ can significantly affect membrane potential and bursting. (C Stokes and J Rinzel)

Goemetric analysis of bursting. Dynamical systems methods and geometric singular perturbation theory are used to analyse mathematical models for bursting oscillations in pancreatic beta cells. We have extended previous work on the fast-slow time decomposition of the equations by allowing for two, not just one, slow negative-feedback variables. New numerical methods are developed in order to understand the underlying bifurcation structure of the fast and averaged subsystems. This has allowed us to classify the mathematical mechanisms responsible for bursting solutions and continuous spiking. We have identified parameter ranges where bistability of both bursting and continuous spiking must exist. Moreover, we have given a detailed description of how chaotic dynamics must arise during the transition from bursting to continuous spiking as parameters are varied. Through the transition, the system must undergo a large number of bifurcations. After each bifurcation, the system exhibits increasingly more chaotic dynamics. (P Smolen, J Rinzel and D Terman: Ohio State U)

Renal Physiology

Ammonia accumulation in the renal medulla. Previously, we described a canonical tubule model for solute, flow, and charge conservation including individual reactive species and chemical buffers. We have now extended the model to include a number of distinct segments of the descending limb of Henle's loop and an interstitium. Ammonium accumulation in the renal medulla is thought to involve countercurrent multiplication, which requires net ammonium secretion into the outer medullary descending limbs. To study how secretion could occur, we used the model to design experiments for measuring permeabilities in the chinchilla. Simulation results suggest the presence of endogenous carbonic anhydrase in the PST2 segment, and we conclude that the long descending limb upper (LDLu) is permeable to both NH3 and NH4. At physiologic pH, the majority of total ammonia entry can be expected to be NH4. However, NH3 entry also occurs and could account in part for luminal alkalinization along the descending limbs. NH4 secretion into the LDLu coupled with active NH4 absorption in the thick ascending limb (established previously) creates an NH4 recycling pathway which theoretically could explain accumulation of ammonium in the medulla. (R Mejia, M Flessner and M Knepper:NHLBI)

Role of the kidney in acid/base balance. We have continued the development of a multinephron model for acid/base balance in the whole kidney, as experiments to measure the necessary parameters continue. A numerical algorithm that uses domain decomposition with parameter continuation to solve a system of differential-algebraic equations with 4th order accuracy in the nephrons has been developed. We have shown that the model is sensitive to the phosphate permeability of the thin descending limbs; a parameter that had not been of much concern previously. For rat data, model permeability larger than 0.00042 cm/s is required to avoid precipitation of CaH2PO4 in the lumen. (R Mejia and M Knepper:NHLBI)

Microcirculation.

Vasomotion. We have revised our recently hypothesized direct relatioship between calcium activation and force-motion generation. Earlier hypotheses in the literature, based on a viscoelastic model or on chemo-mechanical transduction, were found either unsuccessful or incomplete. Our mathematical model uses the experimental force-length and velocity-force relationships for the smooth muscle wall dynamics; the formulation incorporates the load-determining velocity (Podolsky). A description of the mechanics of a thick walled cylinder under intraluminal pressure, with wall viscoelasticity, and time varying wall muscle force has been completed. Intraluminal pressure influences arterial muscle wall activity (myogenic behavior) in the model by having stress shift the activation function for a voltage-gated calcium current. Simulations agree with experiment (Harder et al.) in the dependence of vasomotion activity, arterial radius changes, and membrane depolarization on intraluminal pressure. (J M Gonzalez-Fernandez and B Ermentrout: Univ Pittsburgh)

Selected Other Topics.

Calcium waves of egg fertilization. The egg of the fresh-water fish Medaka exhibits a large calcium wave upon fertilization, which propagates from the point of sperm entrance to the opposite pole. One mechanistic model for the wave (G. Ehrenstein) is as follows: activation of phospholipase C by sperm led to inositol trisphosphate (ITP) accumulation, which released calcium from the endoplasmic reticulum (ER), then this calcium in combination with diacylglycerol led to activation of additional phospholipase C. Recent experimental data required us to refine this model. At least four molecules of ITP must bind to release calcium from the ER. Also a high concentration of ITP (ca. 1 mM) is present in sea urchin sperm at fertilization, and we think in Medaka sperm as well. We have revised the model to incorporate this data and it produces waves consistent with experiment. (P Smolen and G Ehrenstein: NINDS)

Epsilon-entropy and sporadic dynamics. We have pursued the notion of epsilon-entropy, which we used to characterize and classify a variety of complex dynamics. Recently we also applied the idea to nonequilibrium systems exhibiting anomalous diffusion. We proposed that the underlying intermittent or sporadic processes may be identified by a special scaling property of its epsilon-entropy. This is explicitly shown in the case of the Levy random walk, a simple model for anomalous diffusion (as Brownian motion is a generic model of ordinary diffusion). (X J Wang and P Gaspard: Free Univ of Brussels, Belgium)

Muscle. For studying early transients in the onset of striated muscle activity, two experimental setups have been used. One measures length changes resulting from a sudden change in applied force, the other measures force changes due to a sudden imposed change of length. No clear understanding of the mechanism responsible is available. Podolsky suggested that a common underlying mechanism should be responsible for the two types of responses. We have developed a mathematical description based on some considerations of the muscle cross-bridge dynamics that accounts for the two experimental responses.(J M Gonzalez-Fernandez and R Podolsky: LPB)

Morphogenesis. In a certain stage of the asexual cell division cycle of the sacaromyces cerevisiae, a concentrically growing septum develops that leads to the separation between mother and daughter cell. A mathematical representation for the origination and further growth of the septum was formulated in terms of cell membrane geometry and transmembrane enzyme activity. The numerical results obtained reproduce the growth of a planar septum. The use of a window averaging has helped to overcome numerical unstabilities. The problem being considered at this time is how to define a long time shape controling function.(J M Gonzalez-Fernandez and M Karlowitz: Wagner Associates)

Cell Energetics. A reaction-diffusion model for ATP and its byproducts that consists of a symmetric spherical cell with mitochondria distributed in its interior and Na-K pumps at its periphery has been described previously and used to study concentration profiles in a renal cell during transitions in ion transport. Recent structural data for MDCK cells were used to develop a model with cylindrical geometry, radial symmetry and a variable distribution of mitochondria. Initial simulations show large resting ADP gradients over the cell, suggesting that no barrier to ADP diffusion arises as the cell is stressed. On the other hand, [ATP] gradients are less than 10% at rest, so that ATP diffusion is possibly restricted. (R Mejia and R Lynch:U of Arizona)

Solving reaction diffusion equations by Monte Carlo methods. We simulate diffusion with randomly walking elements and reaction with replication and annihilation of the elements. The algorithm belongs to the family of "random gradient method" (RGM), so-called because the density of computational elements represents a derivative of the solution. Integrating to obtain the solution substantially reduces the statistical fluctuations. The method is also adaptive because the computational effort is concentrated where the solution changes rapidly, and is thus suitable for problems with sharp traveling wave fronts, such as nerve or cardiac conduction. We have now extended the technique from one space dimension to two or more. Recovering the solution from a discrete gradient representation is essentially equivalent to computing the forces in an N-body gravitational system. We thus exploit a new fast multipole algorithm, due to Greengard and Rokhlin, which computes the N**2 interactions in O(N) calculations. It may also be possible to employ a gradient representation in deterministic algorithms, replacing the partial differential equation with a system of ordinary differential equations for the locations of the computational elements. A 1-D version has been implemented, and the extension to 2-D is in progress. (A Sherman and M Mascagni)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 13,001-18 MRB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mathematical formulations and analysis relevant to experimental neurophysiology.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. Rall	Senior Research Physicist	MRB, NIDDK

COOPERATING UNITS *(if any)*
Dept. of Neuroscience, Hebrew Univ. of Jerusalem

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

RESEARCH AREA. Basic neuroscience involving structure/function relations for neuronal dendritic branching, dendritic spines, and synapses (also neuron populations, with cortical symmetry), and for such functions as synaptic transmission, amplification and dendro-dendritic interactions in the context of spatio-temporal input patterns, logical processing of input, and neural plasticity, as in conditioning and learning.
RATIONALE. Combine experimental data from neuroanatomy and from electrophysiology with biophysical models of nerve membrane (passive, synaptic and excitable) into a comprehensive theory which can lead to new insights and to testable theoretical predictions (leading to the design of better experiments). To do this we must create, explore and test mathematical and computational models with different degrees of complexity.
METHODOLOGY. Our methods include both analytical solutions and computational solutions of boundary value problems (for partial differential equations) in the tradition of classical physics. They include also the formulation and solutions of problems in terms of systems of ordinary differential equations; when this is done explicitly for a compartmental model of a neuron, it is possible to accommodate a remarkable variety of dendritic branching patterns and non-uniform distributions of membrane properties and of synaptic inputs.
RESULTS. Summarized in Chpt. 3 of "The Handbook of Physiology: The Nervous System, Vol. 1" American Physiological Society, 1977 (Kandel, Brookhart & Mountcastle, eds.), Chpts. 22, 24 in "Cellular Mechanisms of Conditioning and Behavioral Plasticity" (eds. Woody, Alkon, & McGaugh) Plenum Press, 1988, and in Chpt. 2 in "Methods in Neuronal Modeling" (eds. Koch & Segev) MIT Press, 1989.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 13,002-19 MRB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mathematical description of substrate transport to capillary-tissue structures.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. M. Gonzalez-Fernandez	Research Mathematician	MRB, NIDDK

COOPERATING UNITS *(if any)*

Dept. of Mathematics
Univ. of Pittsburg

Lab. of Chemical Biology, NIDDK

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.5	.03

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this work is to develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models of the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. A model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined. A description of the origin and control of vasomotion is being formulated, and applied to the study of microcircution networks.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 13,004-18 MRB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mathematical description of cellular neuroelectric signal transmission.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Rinzel	Chief, MRB	MRB, NIDDK	
Others:	A. S. Sherman	Senior Staff Fellow	P. Frankel	pre-doc ITRA
	C. Stokes	NRC Fellow	M. Rush	pre-doc ITRA
	X. J. Wang	Fogarty Fellow	D. Terman	Temp
	P. D. Smolen	NRC Fellow		
	All in MRB, NIDDK			

COOPERATING UNITS (if any)
Lab/Cell Biol & Genetics, NIDDK;
Dept/Chem, U/California, Davis;
Dept/Mathematics, Arizona St. Univ.
Dept/Mathematics, Ohio State University
Dept/Mathematics, UNAM, Mexico City, Mex.
Med. Neurology Br, NINCDS

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Mathematical Research Branch

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	3.75	.5

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of electrical activity of neurons and other cell types. Among the topics of current interest are: (i) integration of synaptic input delivered to the soma and dendritic branches of a neuron; (ii) propagation of action potentials along axons; (iii) stimulus-response and threshold properties for repetitive-firing of action potentials; (iv) complex bursting patterns of membrane potential oscillations which arise through endogenous membrane properties and/or intercellular coupling.

Because qualitatively related mathematical or biophysical problems may arise in other context, e.g. chemical and biochemical oscillations, or e.g. excitation-secretion coupling, this project may consider models from such applications.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods drawn from both classical and modern applied mathematics. These methods may include finite difference or finite element numerical integration, bifurcation theory, perturbation techniques, and nonlinear dynamical systems theory. One goal of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet physiologically reasonable, equations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 13,020-02 MRB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electrical and Chemical Oscillations in Coupled Cell Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Sherman	Senior Staff Fellow	MRB, NIDDK
Other:	J. Rinzel	Chief, MRB	MRB, NIDDK
	C. Stokes	NRC Fellow	MRB, NIDDK
	M. Mascagni	Guest Worker	MRB, NIDDK

COOPERATING UNITS *(if any)*

Lab/Cell Biol & Genetics, NIDDK

LAB/BRANCH

Mathematical Research Branch

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.48	1.45	.03

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We use mathematical models to study the mechanisms of oscillatory electrical activity arising from ion channels in cell membranes and modulated by intracellular chemical processes. We are interested in both the behavior of single cells and the ways in which cells communicate and modify each other's behavior.

Our main application has been to the biophysical basis of insulin secretion in pancreatic beta-cells. We have examined bursting oscillations in membrane potential and the role of electrical coupling between cells in the islet of Langerhans. Long term goals are to understand how the membrane dynamics interact with intracellular events to regulate secretion and to generalize to other secretory cells and neurons.

Our primary tool is the numerical solution of ordinary and partial differential equations. We use analytical, geometrical, graphical, and numerical techniques from the mathematical theory of dynamical systems to help construct and interpret the models. Perturbation techniques are used to get analytical results in special cases.

We study both detailed biophysical models and simplified models which are more amenable to analysis. Such an approach aids the isolation of the essential or minimal mechanisms underlying phenomena, the search for general principles, and the application of concepts and analogies from other fields.

We see a role for our group as intermediaries between the mathematical and biological disciplines. This includes disseminating the insights of mathematical work to biologists in accessible language and alerting mathematicians and other theoreticians to new and challenging problems arising from biological issues.

ANNUAL REPORT OF THE LABORATORY OF CELLULAR AND DEVELOPMENTAL BIOLOGY

The thirty five members of LCDB focus on study of the critical elements of development and differentiation: how extracellular signals are translated, through transducers and second messengers, into differential gene expression in the nucleus, and how the architecture of nucleic acids and proteins is involved in nuclear and cytoplasmic function. This is an exciting area of modern biology and one in which members of the laboratory are widely recognized as key players.

Several noteworthy recognitions have come to laboratory personnel this year. Dr. Robert Scow was awarded the degree of Doctor of Medicine *honoris causa* by the University of Umea, Sweden, recognizing his years of contributions to understanding lipid transport and the role of lipoprotein lipase. Dr. Robert Simpson received the Public Health Service Meritorious Service Award. Dr. Jurrien Dean was awarded the Commendation Medal of the PHS; he was also named Chief of the newly established Mammalian Developmental Biology Section in LCDB. Dr. Dean Londos was co-organizer of a highly successful Keystone Symposium on biology of the adipocyte. Ms. Dorothy Banks was NIDDK Employee of the Month in recognition of her contributions to the laboratory and to Institute EEO and women's issues programs, particularly her role as an EEO Counselor. An unusual accolade to the work of Dr. Robert Hartley in cloning and expressing a bacterial ribonuclease was the picture of male sterile tobacco flowers on the cover of *Nature*; male sterility was generated by cloning the barnase gene under control of a tapetum-specific promoter in the plants by a group in Belgium.

The members of LCDB study a wide variety of experimental systems and problems. We feel that our diversity adds strength to each individual research program with complimentation of our own expertise by that of others with related interests. In order to emphasize the continuity of our efforts, I review the progress in the laboratory over the past year in a thematic fashion, rather than by the work of each individual section or working group. We will consider chromatin structure and the repression/activation of gene expression; the structure of cell type specific genes; signal transduction from membrane to nucleus; cell biology, genetics and morphology of lipid processing and trafficking of lipid metabolizing enzymes; enzymology of other proteins; and biotechnology.

Chromatin structure: repression of transcription

Nucleosome positioning has been suggested to be a possible mechanism in determining the function of *cis*-acting elements in eukaryotic cells. Previously, we showed that movement of a *cis*-acting element necessary for yeast replication origin function into the center of a nucleosome markedly diminished its accessibility to *trans*-acting factors. Similarly, we showed that a yeast repressor organized positioned nucleosomes abutting its operator, placing the TATA box in the center of the nucleosome, and suggested that this might be the mechanism of repression of the a-cell specific genes which are regulated by the $\alpha 2$ repressor protein.

We have extended these studies, showing that a positioned nucleosome is located 13-16 bp downstream of the $\alpha 2$ operator in a plasmid and two genomic contexts. Nucleosomes are not positioned upstream of the operator in the genome, raising questions about how a symmetric operator, interacting with two homodimeric proteins, creates a polar chromatin structure. The precision of location of the nucleosomes, together with other considerations, suggested to us that $\alpha 2$ might interact with a nucleosome constituent to organize chromatin structure. We have confirmed this supposition, showing that amino-terminal region deletions of histone H4 (del 4-14, del 4-19 and del 4-23), or certain point mutant substitutions in the same region (K16G, R17G and H18G), lead to abolition of the precise positioning observed for wild type histone H4. Examination of the topological properties of plasmids containing the $\alpha 2$ operator in α and a cells leads to results consistent with the minichromosome having one more nucleosome when the $\alpha 2$ repressor is present and an organized chromatin structure results.

The suggestion that chromatin organization might be the basis for repression by $\alpha 2$ was supported by studies which show that the expression of a *STE6*-β-galactosidase reporter gene was derepressed in a-cells when H4 mutants that abolish positioning were expressed; this was true at both the protein and mRNA levels. Similarly, another a-cell specific promoter coupled to a reporter gene was derepressed in yeast expressing the H4 amino terminal region mutants. In this case, expression was high in both aaa and $\alpha\alpha\alpha$ cells, eliminating the possibility that the results were an artefact of the pseudodiploid state of the H4 deletion cells. Similarly, the glycyl point mutants of residues 16, 17 or 18 had derepressed expression of the reporter gene. A mutation of K16 to glutamine neither led to disruption of nucleosome positioning on the *STE6* promoter nor allowed expression of the reporter gene. In all cases examined, the presence of a positioned nucleosome adjacent to the $\alpha 2$ operator correlated perfectly with repression of the gene, providing strong support for the contention that chromatin structure may be causal in control of transcription of the a cell specific genes.

$\alpha 2$ serves another function in yeast gene regulation. As we showed previously, $\alpha 2$ forms a heterodimer with a1, another homeodomain containing protein, to bind to operators that repress haploid specific gene expression in diploid yeast cells. Methylation interference experiments have now shown that protein-DNA contacts span three successive major and two minor grooves of the nucleic acid. We have investigated the sequence requirements for this interaction and find that the minor groove contacts in the middle of the operator can be eliminated without affecting binding *in vitro*. The effect of mutations in the flanking regions of the operator is now under investigation *in vitro* and *in vivo* using expression of a linked reporter gene. Comparison of these data with those for an $\alpha 2$ operator, which suggest that most mutants, while not binding $\alpha 2$ *in vitro*, do not affect repression by $\alpha 2$ *in vivo*, will be of interest.

The relationship of nucleosome positioning and chromatin structure to transcription has also been studied for POL III genes. Studies detailed above suggest that chromatin structure might be critical in repression of transcription by RNA polymerase II. Other studies during the past year have dealt with transcription of tRNA and 5S RNA genes. Studied in a multicopy plasmid, deletions in the 5'-flanking region of the 5S RNA gene reduce *in vivo* synthesis of 5S RNA. Similarly, in constructs without deletions, moving the same region into a predicted positioned nucleosome also reduces transcription; the data suggest that occlusion of the TFIIIB binding site by interactions with histones in a nucleosome negatively affects transcription. These studies will be extended to examine the other end of the 5S gene, where TFIIIA binds, and by structural studies of the various constructs.

Another POL III gene, that for tRNA, behaves differently in the context of chromatin structure and transcription. A tRNA gene was fused to two different nucleosome positioning signals in the TRP1ARS1 plasmid in a fashion such that the predicted nucleosome position would include the tRNA start site and essential A box element near its center. Surprisingly, transcription of the tRNA gene was not affected *in vivo* and the predicted nucleosomes were not positioned over the genic elements. The predicted nucleosome positions were observed for tRNA sequences mutated so as to be inactive. The suggestion from these results *in toto* is that there is a competition in the cell between transcription factors and histones - for POL III factors and tRNA genes, transcription wins, but for POL III and 5S RNA or POL II, positioned nucleosomes can abolish transcription.

Chromatin structure: activation of transcription

Activation of transcription has recently been a favorite topic of the molecular biology community; *trans*-acting factors have been identified or isolated for many controlled domains. Several candidate factors have been implicated in control of genes under study in this laboratory. We have studied the regulation of the *HSP82* gene during sporulation in yeast, for example. The gene is developmentally regulated; a plasmid containing 2.2 kb of upstream sequence fused to a reporter gene is induced in diploids, but not haploids, on starvation. Insertion of two fragments of the upstream sequences, 200 bp centered around the heat shock element (HSE) and 130 bp about 1 kb upstream containing numerous potential regulatory factor binding sites, adjacent to a *CYC1*/lacZ reporter leads to high levels of basal expression, but no induction on sporulation. When only the HSE sequences are present, basal expression is decreased five-fold but *HSP82* mRNA is increased five-fold on starvation in diploids, but not haploids; the level of expression is only about 10% of that seen for the 2.2 kb controlling sequence. The HSE element appears to confer diploid specificity for expression while the further upstream sequences are necessary for high level transcription.

Our studies of *trans*-acting factors activating transcription extend to two mammalian systems. The human ϵ-globin gene is the first of the β-globin like genes to be expressed during development. Transcription occurs between the 6th and 10th weeks of fetal life; the gene is also expressed in the human K562 leukemia cell line, the subject of our investigations. We have studied DNA protein complexes which may be involved in generation of an active chromatin conformation about the ϵ-globin gene. We have now completed a detailed map of *in vitro* nuclear factor binding sites in the promoter of this gene. We used a combination of DNase I footprinting and gel mobility shift assays with nuclear protein extracts from erythroid and non-erythroid cells. Both single and competitive interactions were found, including the erythroid factor, GATA-1, which bound to three sites in the promoter and Sp-1, which bound to the CACCC motifs.

These *in vitro* studies were complemented and extended using electroporation to transfect a vector containing the CAT reporter gene driven by the ϵ-globin promoter into K562 and HeLa cells. The chicken β/ϵ-globin 3' enhancer greatly increased transcription of the CAT gene from the ϵ-globin promoter, exclusively in erythroid cells. Mutational analysis of the promoter showed that transcriptional response to the enhancer was dependent on the GATA-1 binding site at -165 (a site which is conserved in the promoters of ten mammalian globin genes expressed during embryogenesis); mutation of the GATA-1 site at -210 had virtually no effect on transcriptional activity.

The chicken enhancer employed in the above studies contains duplicated GATA-1 and AP-1 motifs, which have been shown by others to be critical for transcriptional activation. In K562 cells, we have now shown that the enhancement of transcription from the ϵ-globin promoter involves the two AP-1 binding sites. We have also shown that a fragment of the human β-globin locus controlling region, which activates transcription from the ϵ-globin promoter by over 100-fold, also resides in duplicated AP-1 sites, and not in the single GATA-1 binding motif.

To extend these results into cells, we have constructed an episomal vector system, based on the EBV origin of replication, which contains a 5 kbp segment of human DNA including the ϵ-globin gene, marked in the 5' non-coding region. The construct has been cloned into K562 and HeLa cells, which exist as stable transformants with 2-150 copies of the episome. Some of the clones produce low, but measurable, amounts of ϵ-globin mRNA from the episome. Modification of the episomal DNA, or culture conditions, will be our next step in trying to achieve regulated transcription of the gene *in vivo* and allow structural studies of active and inactive genes in mammalian chromatin.

The mammalian zona pellucida, an extracellular coat which surrounds the growing oocyte, mediates species specific sperm interactions leading to fertilization, and protects the growing embryo prior to implantation, has been a research subject in LCDB for several years. Details of the organization of the genes for the zona proteins elucidated in this laboratory are reviewed below. In the context of transcriptional control, we have identified five short DNA elements (I, IIA, IIB, III and IV) in the 5'-flanking regions of human and murine *ZP-2* and *ZP-3* genes that are relatively conserved in both sequence and location.

Deletion analysis of the 5'-flanking region of the mouse *ZP-3* gene ligated to a luciferase reporter gene and injected into growing oocytes suggested that element IV (the most distal conserved sequence) was critical for tissue specific expression. This suggestion was confirmed by findings that a six bp mutation in the center of element IV markedly inhibited expression of the reporter gene and that element IV alone, upstream of a minimal *ZP-3* promoter, markedly enhanced expression. Taken together, these observations suggest that element IV is both necessary and sufficient for *ZP-3* expression in murine oocytes; other DNA and protein species may, of course, be necessary for the level of expression observed *in vivo*.

Element IV is a 12 bp sequence that is 75% identical among the four mouse and human genes studied. Oligonucleotides containing either the *ZP-3* or the *ZP-2* element IV form protein-DNA complexes of identical mobility with lysates from oocytes in gel mobility shift assays. The protein-DNA complex is detected only in ovarian tissues, in an organ survey, and, within the ovary, was present only in oocytes and not in follicle cells. We assume that a common protein (or protein complex) recognizes the core sequence of element IV, $\mathrm{CAC^{G}_{C}TG}$, and has a stimulatory effect on transcription of the zona gene family. Since the putative protein regulator is present only in oocytes, it seems likely that it plays a role in the temporal and spatial specific expression of the zona genes. Efforts to clone the gene for the regulatory protein are actively underway; success in this endeavor should allow a clear resolution of this possibility.

Genes for cell type specific proteins

We have previously characterized the *ZP-3* genes from mouse and human and the *ZP-2* gene from mouse. We have now screened a human genomic library and isolated recombinant clones which include the coding sequence for human *ZP-2*. It is a single copy gene which spans 12.3 kbp. Eighteen exons, ranging in size from 45 bp to 190 bp are almost identical in size to the 18 exons of the murine gene. Nucleic acid sequence of the coding regions is 72% identical between the two species and the predicted protein products are 60% identical in amino acid sequence. As for *ZP-3*, there is a striking resemblance in hydropathicity profiles for the *ZP-2* proteins in man and mouse. Using oligo dT and oligonucleotides based on the sequence of exon 1 of human *ZP-2*, we have isolated a full length human cDNA for this gene from ovarian poly(A)$^+$ RNA using the polymerase chain reaction. Similar to the other three cDNAs, this has short 5' and 3' untranslated regions. The human *ZP-2* cDNA has a single open reading frame of 2145 nt encoding a 715 amino acid protein, two amino acids longer than the murine homologue.

A surprising observation made this year is the occurrence of a second human *ZP-3* allele. An additional G leads to a frame shift mutation near the carboxyl terminus of the protein and introduces a termination codon which truncates the protein by 52 amino acids. The resultant protein differs strikingly in lacking a hydrophobic region which is present in the other human allele as well as the mouse and hamster proteins. The second allele is present at high frequency in the human population. Ongoing studies will address the question of whether the second allele, encoding the truncated protein, has an adverse effect on human fertilization and early embryonic development.

Perilipin is a fat cell specific protein which is intimately associated with the surface of lipid droplets, as discussed in more detail below. We have isolated cDNA clones for perilipin from a rat adipocyte library. The gene is expressed as a major 3 kb message with a minor 3.6 kb band. It is estimated to comprise about 0.1% of messenger RNA when maximally active. An open reading frame codes for a predicted protein of 550 amino acids, in good agreement with the size of perilipin on denaturing gel electrophoresis of about 60000 daltons. Several peptides sequenced from isolated perilipin are found in the predicted amino acid sequence, confirming the identity of the cloned DNA. The protein has no similarity to other proteins in the data bases, has no transmembrane domains or N-linked glycosylation sites but does have five potential phosphorylation sites for A-kinase, consistent with its phosphorylation *in vivo*. We are in the process of isolating genomic clones for perilipin from rat and human libraries.

Expression of perilipin mRNA was detected in rat, mouse and human adipocytes; the gene was not expressed in a variety of other mammalian tissues, including liver and muscle. As model systems for study of the control of perilipin synthesis, we have used differentiating adipoblasts and isolated adipocytes. 3T3-L1 fibroblasts differentiate into adipocytes on treatment with dexamethasone, accumulating triacylglyceride in lipid droplets. Perilipin mRNA accumulates in parallel with triacylglycerol during differentiation. The large fat droplet characteristic of mature adipocytes is not observed when differentiation takes place in the absence of biotin; minuscule lipid droplets scattered throughout the endoplasmic reticulum, associated with perilipin, are observed instead. Normal amounts of perilipin mRNA and protein accumulate in these cells, however, suggesting that perilipin expression and abundance are coupled to lipid surface area, not lipid mass. Isolated white rat adipocytes in long term culture show a rapid disappearance of perilipin mRNA on establishment followed by a gradual reappearance of the message after 24 hours. Over the same time period, cells switch from an insulin sensitive state to a state unresponsive to the hormone, as indicated by the subcellular localization and abundance of the glucose transporters Glut1 and Glut4. These systems are being employed to examine the effects of adipogenic factors and nuclear transcription factors on the expression of perilipin and other adipocyte specific genes.

Signal transduction: from the membrane to the nucleus

Changes in gene activity are the ultimate result of environmental signals which lead to differentiation of cells. A favorite system in this laboratory for investigation of the mechanisms of differentiation is the slime mold, *Dictyostelium discoideum*. We have previously shown that cAMP, a multifunctional differentiation signal involved at several stages of the life cycle of *Dictyostelium*, has several different mechanisms whereby it influences expression of specific genes. In a long term effort to reconstruct the molecular mechanism whereby cAMP, acting as an extracellular differentiation factor, leads to altered gene expression, we have recently concentrated on early events in the signal transduction pathway. We have isolated and characterized genes which code for four different cAMP receptors in *Dictyostelium*, cAR 1-4. While other potential receptor genes have been identified by hybridization analysis, they are but distantly related to these four genes, which seem to comprise all the members of the cAMP receptor family. The cAMP receptors are members of the group of cell surface receptors which interact with G-proteins and are characterized by having seven membrane spanning domains. The transmembrane and loop regions are closely related (about 60% identity in amino acid sequence) among all four cAMP receptors but the amino and carboxyl terminal regions of the proteins are quite dissimilar. Specifically, the carboxyl terminal tail of cAR1 possesses several clusters of seryl residues, while the intracellular carboxyl terminal regions of cARs 2, 3 and 4 lack such clusters.

As might be expected in an organism which relies on different concentrations and pulsations of cAMP for morphogenesis, the cARs have temporally and spatially specific modes of expression and/or control in *Dictyostelium.* Both cAR1 and cAR3 are expressed during early development; this is the time when signalling and chemotaxis are active and cells respond to cAMP pulses. The expression of cAR1 requires cAMP signalling while cAR3 can be induced in the absence of such signalling. cAR1 protein is phosphorylated with kinetics that precisely parallel the desensitization of cAMP receptors which are coupled to adenylyl cyclase. The seryl clusters in the intracellular carboxyl terminal domain of cAR1 are likely candidates for the phosphorylations associated with down regulation. The association of a specific cAMP receptor with chemotaxis is less clearly defined; the observation that chemotaxis persists in mutant *Dictyostelium* which express cAR3 but lack cAR 1, 2 and 4 makes cAR3 a strong candidate for the chemotactic receptor.

Although the time course of maximal expression differs for all four receptor genes, they are all expressed at some level in late developmental stages, after cytodifferentiation and at the time when cells respond to the continuous presence of extracellular cAMP. Each cAR has a distinctive spatial distribution of expression, although there is a general tendency for expression to be greater in prestalk than prespore cells, consistent with cAMP binding results and signalling experiments suggesting greater sensitivity to cAMP in the prestalk region of developing aggregates. Appearance of cAR2 and cAR4 messages at the time in development when cells alter their sensitivity to cAMP suggests a relationship of these receptors to cell type specific gene expression and/or morphogenetic movement.

Reverse genetics is now well established for *Dictyostelium*, allowing detailed questions about the role of the various cARs to be asked. Constructs have been prepared to generate null mutations for each receptor for analysis of their effects on development. Promoter elements have been identified in the genes for each cAR and study of the factors and signals regulating their expression during development are beginning. Given the family nature of the cAR genes, particularly interesting experiments are possible using chimeric constructs which will create either fusion proteins containing domains of different receptors or genes which will express a different receptor protein under the control of a particular temporally and spatially regulated promoter for another cAR.

On the road from the cell surface to the nucleus, we have begun to clone the next players in signal transduction. Previously, we identified a putative G protein clone in *Dictyostelium.* We have now isolated cDNA clones for eight different probable Gα proteins for further study. Coexpression experiments to identify linkages between specific cAMP receptors, Gαs and distal effectors are anticipated or in progress.

The isolated adipocyte has a long history of utility as a model for molecular endocrinology, particularly signal transduction from membrane hormone receptors through G proteins and adenylyl cyclase and other terminal effectors. Much of this history occurred as a result of the work of members of LCDB, originating with the isolation of fat cells by Dr. Martin Rodbell. We have previously reported cAMP independent effects of insulin on lipolysis and glucose transporters in fat cells and suggested that these might be mediated by protein kinase and phosphatase activities. We also reported that insulin rapidly activates protein kinase C and stimulates a phospholipase C activity in adipocytes. The involvement of protein kinase C in insulin action has been a matter of contention, particularly since a prominent protein kinase C substrate is phosphorylated in many cell types in response to phorbol esters but not to insulin. We have now analyzed the protein kinase C isozyme population in adipocytes and adipocyte plasma membranes in the basal state and after exposure to phorbol esters, insulin or vasopressin. Through the use of isozyme specific monoclonal antibodies, we can show that adipocytes contain at least five of the known isozymes. Measurement of activities in plasma membranes suggests that different isozymes are activated by phorbol esters and by insulin; phorbol ester stimulation produces an isozyme stimulation profile far more similar to that produced by vasopressin than that deriving from insulin stimulation. The data indicate some degree of isozyme specificity for protein kinase C translocation to plasma membranes as a function of the stimulatory agent; the specific isozymes activated by the various agents remain to be identified.

Cell biology of lipids, lipases, and lipid-associated proteins

The surface of the lipid droplet in adipocytes is the site of deposition and retrieval of stored lipid; little is known of the architecture and mechanisms of reactions which occur at this critical juncture. We detail above advances made in understanding of the gene for perilipin, a protein which we initially identified as the major cellular target for phosphorylation when cAMP concentrations are elevated in adipocytes. We have now obtained light and electron microscopic evidence which strongly suggests that this hormonally regulated, adipocyte specific protein is located at the surface of the lipid droplet.

Using 3T3-L1 fibroblasts stimulated to differentiate into adipocytes as a model system, we studied the location of perilipin using electron microscopic immunocytochemistry and specific stains for hydrophobic lipid and endoplasmic reticulum. Cells grown with biotin had large lipid droplets surrounded by endoplasmic reticulum. Cells grown in the absence of biotin had small lipid droplets aggregated in irregular patches in the cytoplasm; endoplasmic reticulum was present in these patches. For both large and small lipid droplets, endoplasmic reticulum membrane leaflets were continuous with the monolayer in contact with the lipid droplet core. Immunogold labeling of perilipin was found on the surface phospholipid monolayer of the lipid droplets and also within cisternae of the surrounding endoplasmic reticulum. Hormonal regulation of the phosphorylation state of perilipin together with its unique subcellular localization suggest a role for this structural molecule in lipolysis, perhaps as a pore component or a docking site for hormone sensitive lipase.

Two other lipases have been localized using similar morphological techniques. Hepatic lipase is believed to be synthesized in hepatocytes yet have its site of action in hydrolysis of chylomicrons and VLDL remnants at the endothelial surface. Two different antisera lead to different locations for hepatic lipase. One shows immunogold labeling between the basal surface of hepatocytes and the sinusoidal epithelial cells, in the extravascular space of Disse. The other locates lipase associated with the sinusoidal endothelium, but intravascularly. This puzzling result may result from different forms of the lipase specific to each site, in spite of the fact that the antisera are polyclonal. Given the high antigenicity of carbohydrate chains, it is possible that oligosaccharide modifications of the lipase may differ for the two populations and be reflected as the different epitopes detected.

Both hepatic and lipoprotein lipase activities are deficient in homozygous *cld/cld* mice; this mutation leads to massive hypertriglyceridemia and death within a few days of birth. Hepatic lipase was present extravascularly in liver and extracellularly in hepatocyte cultures from both normal and *cld/cld* mice. In contrast, lipoprotein lipase was present intracellularly in liver and hepatocyte cultures of *cld/cld* mice, but not those of normal mice. Intracellular processing of the two glycoproteins differs in mice bearing the genetic lesion.

We have pursued studies of the glycosyl modification of both lipases in normal and *cld/cld* mice in an effort to elucidate features of the biochemistry of this genetic defect. Lipoprotein lipase is synthesized by parenchymal cells and transferred to capillaries; it is thought to be a dimer of identical glycoprotein subunits. Production of lipase involves synthesis and glycosylation of subunits in endoplasmic reticulum, trimming of glucose and mannose residues from N-linked endo H-sensitive oligosaccharide chains in the reticulum and Golgi, addition of other kinds of sugars to the chains in

Golgi, dimerization of subunits and secretion of lipase with endo H-resistant (complex) type oligosaccharide chains.

Brown adipocytes from *cld/cld* mice in culture synthesize glycosylated lipoprotein lipase, but the enzyme is inactive, incompletely processed and retained in endoplasmic reticulum. Some data suggested that the lipase might exist as a dimer, but sucrose gradient centrifugation showed that the protein was an aggregate of monomers. The molecular weight of the endo H-sensitive subunits of lipase from *cld/cld* cells is about 2000 daltons less than that of lipase from cells treated with castanospermine to block the action of glucosidase I, suggesting that a subnormal number of saccharide residues may be added to lipase in these cells or that trimming of the oligosaccharide chains proceeds beyond the usual action of glucosidase I. Whether the *cld/cld* mutation affects synthesis or processing of oligosaccharide modifications of lipoprotein lipase in the endoplasmic reticulum, transport of lipase from reticulum to Golgi or some other feature in production of the enzyme remains to be resolved. A further problem in understanding the basis of this genetic lesion is the difference in metabolism of the two lipases affected, noted above.

We have also used ultrastructural methods to further refine the defect in lipid transport in Niemann-Pick Type C disease, under study in the laboratory for several years. Freeze fracture sections labeled with filipin show a characteristic deformation where the probe interacts with cholesterol, enabling quantitation of its distribution. The highest density of membrane deformations in normal fibroblasts was in Golgi condensing vacuoles while most deformations in diseased fibroblasts were in Golgi trans cisternae. Quantitation of the two situations suggests that translocation of cholesterol to condensing vacuoles is deficient in Niemann-Pick cells, providing a probable explanation for the deficiency in mobilization of cholesterol and its transport to the plasma membrane in Niemann-Pick Type C cells.

Protein chemistry and enzymology

Two proteins, barnase (the extracellular ribonuclease of *B. amyloliquefaciens*) and barstar (its cognate intracellular inhibitor), have been the subject of investigation for many years in LCDB. They are utilized as model systems for protein folding investigations and study of protein-protein interactions in their 1:1 complex. Cloning, expression and mutagenesis have provided powerful tools for study of these problems in the past five years.

The structure of barnase has been refined against 2.0 Å x-ray data; that for barstar in solution is nearing completion using 2-D NMR methods in the laboratory of Dr. Jean Garnier. An exciting development this year has been crystallization of both barstar and the barnase barstar complex in the laboratory of Dr. Yves Mauguen; x-ray solution of their structures is eagerly anticipated. Solution physicochemical studies of the stability of barstar derivatives mutated at the two cysteinyl residues have shown that substitution of alanyl residues leads to a functional protein nearly as stable as the natural protein, allowing study of the protein without the interference of variable degrees of disulfide crosslinking.

We have described before the competition assay using an active and an inactive barnase together with barstar to measure the dissociation rates of barnase barstar complexes and the equilibrium binding of the two barnases. Early experiments using various combinations have yielded relaxation times ranging from minutes to hours. While inactive barnase (mutated to glycine at the active site histidyl residue at position 102) forms a tight complex with barstar, its dissociation constant is three orders of magnitude greater than that for the wild type protein. We have used mutagenesis of surface residues in barnase (80 mutants) and charged residues in barstar (60 mutants) to probe possible interacting faces for the two proteins. Asp 35 and Asp 39 of barstar were previously identified as important for protein protein interactions; Arg 59 of barnase has now been added to the list of residues important for complexation. A double mutant of barnase, R59Q and H102G, does not bind barstar at all. Arg59 is near the active site of barnase, suggesting that inhibition involves simple exclusion of substrate from the active site.

Two enzymes isolated and studied in Eastern Europe bear considerable homology to barnase; binase (from *B. intermedius*) and RNase Sa have provided the basis for collaborative investigations of enzyme structure function relationships. Several mutants of barnase which make it more homologous to one of the other enzymes have been made and are under study in terms of their stabilities and kinetics in collaboration with groups in Moscow and Bratislava.

In addition to work carried out in this laboratory and collaborative projects, nearly twenty laboratories world wide are using the barnase/barstar system for study of general problems of protein folding, stabilization, and interactions. Distribution of the mutants and clones from our own cottage industry has spawned a major effort in the protein structure community using these proteins for structural study. More than a dozen papers have appeared in the past year using barnase and/or barstar for detailed molecular analyses of protein structure.

Dihydrofolate reductase, a critical enzyme in folic acid metabolism and the target for a series of drugs (methotrexate is the propositus), has been under study in this laboratory for many years. We are now using a recombinant form of the human enzyme, expressed in bacteria, in collaborative studies. Modification of the sole cysteinyl residue sulfhydryl group of the chicken liver enzyme leads to a marked increase in enzymatic activity, as does inclusion of moderate concentrations of chaotropic agents in the assay. The human enzyme responds to these agents in a more subdued fashion, with a two fold activation being the maximal observed. This is similar to the response of bovine dihydrofolate reductase.

Applied molecular biology/biotechnology

Certain aspects of work in LCDB come closer to the clinic or research and development as outgrowths of or adjuncts to our basic scientific research. For example, we have previously reviewed efforts to produce an active contraceptive vaccine, efforts which were based on an understanding of the structure of the zona pellucida genes and proteins. These continue, with current attempts to improve the efficacy of the vaccine using dual immunization with epitopes from both *ZP-2* and *ZP-3*. Other work in this area includes study of adipocyte metabolism in cachexia and obesity and the production and development efforts of the Biotechnology Unit.

Previously, we reported that IL-6 inhibited lipoprotein lipase activity in isolated or cultured adipocytes and suggested that this cytokine might play a role in cachexia. An autocrine role for IL-6 was further suggested by finding that tumor necrosis factor (cachexin) stimulates IL-6 production in fat tissue and that 3T3-L1 preadipocytes decrease their production of IL-6 when the cells are exposed to dexamethasone, a differentiation inducing hormone. We now find that addition of exogenous IL-6 inhibits differentiation of preadipocytes in a dose dependent fashion. Addition of antibodies to IL-6, on the other hand, potentiates differentiation of 3T3-L1 fibroblasts to adipocytes. The data suggest an autocrine role for IL-6 in acute and chronic aspects of adipocyte metabolism and provide possible leads into understanding the cachexia of chronic illnesses.

Variations in metabolic properties of regional adipose tissue have been thought to be critical in overall body lipid metabolism - the "pear *vs.* barrel" question. We have studied plasma membrane hormone signalling in lean and obese subjects, testing abdominal and gluteal fat samples. Adenylyl cyclase in isolated plasma membranes from abdominal tissue is more responsive to both stimulatory and inhibitory agents than cyclase from gluteal adipocytes. The regional tissue differences are not seen in adipocytes from obese individuals - their cyclase is consistently of the unresponsive character. A biochemical property of the obese state is preserved in isolated plasma membranes from adipocytes, leading to hope for understanding of the deficits in metabolically disabled obese individuals.

The Biotechnology Unit is an unique facility at NIH. It both provides service to the NIH scientific community in producing large amounts of custom fermentation biomass and the initial, large scale processing of biomaterials, and undertakes research and development work designed to facilitate its service role. In the past year, nearly 200 large scale preparations were performed, involving culture in volumes up to 300 liters of bacteria, recombinant DNA containing organisms, and mammalian and insect cells. Gram quantities of two specific proteins were purified.

A major effort of the Unit for the past few years has been optimization of production conditions for bacterial toxins - these are used to create chimeric therapeutic reagents for trials at selective killing of tumor or other disease (*e.g.* AIDS) specific cells. Specifically, modified toxin molecules based on a recombinant *Pseudomonas* exotoxin A, cholera toxin B subunit, and diphtheria toxin, were purified after optimization of culture conditions for toxin production.

In development work, experiments were conducted to improve existing methods for growth and infection of insect cells in culture harboring baculovirus based vectors for expression of eukaryotic genes. A serum free medium was developed as were methods for attaining high cell density in bioreactors. Nutrient metabolism at the high cell densities achieved under optimal growth conditions was a critical element in effective infection and production of recombinant DNA encoded proteins. Another development effort, possibly applicable to the insect cell culture system in the future, is adaptive control of bacterial aerobic fermentation. The general purpose control algorithm is based on input/output data, without the need for theoretical modeling. This algorithm has proven successful in supporting growth of several different microorganisms to maximal cell density in bioreactors ranging in size from 3 to 300 liters.

This overview of the work of the Laboratory of Cellular and Developmental Biology is a brief summary of the studies carried out in the past year by talented and productive scientists. We hope that the connectiveness of our science and its diversity leads the reader to a sense of the excitement we feel in our daily interactions as we move forward in understanding of the most interesting aspects of cellular and molecular biology in a very exciting time for modern biology.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15100-21 LCDB

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October 1, 1990 to September 30, 1991

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Protein Nucleic Acid Interactions: Chromatin Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

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	Christopher Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

T. Richmond, ETH, Zurich, Switzerland (foreign)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
6.5	6.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

The role of chromatin structure in modulating the functions of DNA in transcription, replication, recombination and repair is becoming increasingly apparent. Nucleosome positioning has been suggested to be a possible mechanism in determining the function of cis-acting elements in eukaryotic cells. Previously, we showed that movement of a cis-acting element necessary for yeast replication origin function into the center of a nucleosome markedly diminished its accessibility to trans-acting factors. Similarly, we showed that a yeast repressor organized positioned nucleosome abutting its operator, placing the TATA box in the center of the nucleosome, and suggested that this might be the mechanism of repression of the a-cell specific genes which are repressed by the alpha2 repressor. We have extended these studies, showing that a positioned nucleosome is located 13-16 bp downstream of the alpha2 operator in a plasmid and two genomic contexts. The precision of location of the nucleosomes, together with other considerations, suggested to us that alpha2 might interact with a nucleosome constituent to organize chromatin structure. We have confirmed this supposition, showing that amino-terminal region deletions of histone H4, or certain point mutant substitutions in the same region, lead to abolition of the precise positioning observed for wild type histone H4. As suggested by this result, we show that the expression of a STE6-beta-galactosidase reporter gene was derepressed in a-cells when H4 mutants that abolish positioning were present. alpha2 forms a heterodimer with a1, another homeodomain containing protein, to bind to operators that repress haploid specific gene expression in diploid yeast cells. Methylation interference experiments have now shown that protein-DNA contacts span three successive major and two minor grooves of the nucleic acid. We have investigated the sequence requirements for this interaction and find that the minor groove contacts in the middle of the operator can be eliminated without affecting binding in vitro. We have studied the regulation of the HSP82 gene during sporulation; the HSE element appears to confer diploid specificity for expression while the further upstream sequences are necessary for high level transcription. A second major interest in this project is the relationship of nucleosome positioning and chromatin structure to transcription. We have studied both POL II and POL III genes, including tRNA and 5S rRNA for the latter enzyme. Results suggest that there is a competition in the cell between transcription factors and histones -- for POL III factors and tRNA genes, transcription wins but for POL III and 5S RNA or POL II, positioned nucleosomes can abolish transcription. Collaborative studies of higher order chromatin structure and the crystal structure of a nucleosome core particle containing unique sequence DNA continue.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15102-31 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Ribonuclease and its inhibitor from Bacillus amyloliquefaciens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Robert W. Hartley Research Physicist LCDB:NIDDK

COOPERATING UNITS *(if any)*

Dr. Yves Maugen, Laboratoire de Physique, Centre Pharmaceutique, Universite de Paris-Sud. Dr. Jean Garnier, Protein Engineering Unit, Biotechnology INRA, Jouy-en-Josas, France. Dr. Guy Dodson, Chem. Dept., U. of York. Dr. Josef Sevcik, Institute of Molecular Biology, Slovak Academy of Science, Bratislava, Czechoslovakia.

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are used as a model system for the study of protein folding and protein-protein interactions. Barnase is one of an homologous group of ribonucleases occurring in both prokaryotes and eukaryotes.

Recombinant DNA techniques are being applied with three major aims: (1) to facilitate production of wild type and mutant proteins; (2) to examine the structural and control sequences of the genes; and (3) to make specific changes in the sequences to test theories of folding and probe the barnase-barstar interaction.

The lethal effect of the cloned wild type barnase gene can be repressed by expression of the barstar gene on the same plasmid. E. coli plasmid vectors have been devised for both proteins and both can now be obtained essentially pure in 100 mg quantities. DNA and amino acid sequences are known for both and the x-ray structure of barnase has been refined to 2.0 Å. The structures of both proteins in solution are being studied by 2-D NMR and x-ray work on both barstar and the barnase-barstar complex are under way. A synthetic fluorescent substrate has been used to study hydrolysis kinetics and to look at the kinetics and stability of the barnase-barstar interaction for native and mutant proteins. A number of variations in the sequence of each protein (80 in barnase, 60 in barstar) have been obtained by oligonucleotide-directed mutagenesis. Some of these were aimed at specific questions but most are part of a survey of the protein surfaces designed to locate their areas of interaction and residues on both have been identified as being so involved.

Our finding that the two Cys residues of barstar can both be replaced by Ala without loss of activity or yield will greatly simplify future studies of barstar folding. Such replacement of either or both of the Cys residues reduces the stability of barstar only to that of the wild-type measured in the presence of mercaptoethanol or DTT.

A method has been developed for measuring the relative strength of the bond between barnase and barstar for various combinations of wild-type and mutant proteins.

Recent work, elsewhere, in which the barnase gene was attached to a eukaryotic promoter in order to kill the tissue in which that promoter is expressed (in the first instance to produce male sterility in plants) has aroused considerable interest in its possible use in developmental studies and as the key to a variety of anti-viral strategies.

U S GOVERNMENT PRINTING OFFICE: 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15200-31 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Studies on Folic Acid (Dihydrofolate Reductase)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.:	Bernard T. Kaufman	Research Chemist	LCDB:NIDDK
Others:	John Bieri	Scientist Emeritus	LCDB:NIDDK

COOPERATING UNITS *(if any)*

Dr. James Freisheim, Medical College of Ohio

LAB/BRANCH

Laboratory of Cellular & Developmental Biology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER.
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

We continue our studies on dihydrofolate reductase (DHFR), a key enzyme in folic acid metabolism and the target site for a series of important drugs that are structural analogs of the vitamin, folic acid. Although the liver is the major site for this reductase activity, initial attempts to investigate DHFR in human liver was deterred by the observation that enzyme activity was extremely low in this tissue. However, the human reductase (hrDHFR) has now been cloned and expressed in E. coli by Dr. James Freisheim, Medical College of Ohio and is available in somewhat large amounts. In a continuing collaboration, I am examining certain activation phenomenon and the properties of a specific sulfhydryl group in the human enzyme such as I previously discovered and studied in DHFR derived from the chick, beef, pork and sheep.

In studies, thus far, the hrDHFR responds to -SH modification in a manner similar to that observed with the beef reductase. About 2-fold activation is noted after treatment with p-chloromercuribenzoate (pcmb). Other organic mercurials exhibit neither activation nor inhibition. This is to be compared with the chicken liver reductase (clDHFR) that is activated by all organic mercurials examined to date. In addition, tetrathionate and iodine that activate clDHFR as much as 5-fold have no effect on the hrDHFR. Again, the maximum transient activation by 4-5 M urea is only about 2-fold, whereas the same urea concentration yields about 5-fold with the clDHFR. Whether this is the maximum activation that can be induced in the human enzyme as compared with the dramatic increases in activity observed with the clDHFR is the basis for current studies. The possibilities of relating these observations to differences in the structure of the various DHFRs, particularly in the region of the -SH that is removed from the active site are being explored. In addition, a hypothesis relating these observations to a potential physiological function is under consideration.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15401-19 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthesis and Transport of Lipoprotein and Hepatic Lipases in Tissues and Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Robert O. Scow	Chief, Endocrinology Sect.	LCDB, NIDDK
Others:	Jin-Woo Park	Visiting Fellow	LCDB, NIDDK
	Charles J. Schultz	IRTA Pre-doctoral Fellow	LCDB, NIDDK
	E. Joan Blanchette-Mackie	Research Biologist	LCDB, NIDDK
	Albert E. Spaeth	Chemist	LCDB, NIDDK

COOPERATING UNITS *(if any)*

Dr. Hiroshi Masuno, 2nd Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu, Ehime, Japan; Dr. Tsuneo Takahashi, Department of Oral Anatomy, Kanagawa Dental College, Yokosuka, Kanagawa, Japan

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.75	1.75	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mice born with combined lipase deficiency (cld/cld) have very low lipoprotein (LPL) and hepatic (HL) lipase activities, develop severe hypertriglyceridemia, and die within 3 days. The recessive mutation (cld) causing the deficiency is located on chromosome 17, whereas LPL and HL genes are on chromosomes 8 and 11, respectively. Structural genes for the lipases are normal in cld/cld mice. Both lipases are normally synthesized and secreted by parenchymal cells and transferred to endothelial cells where they act. The active secretable form of LPL is thought to be a dimer of identical endo H-resistant glycopeptides. Brown adipocytes cultured from cld/cld mice synthesize and glycosylate LPL but it is endo H-sensitive, inactive and retained in endoplasmic reticulum (ER). Immunocytochemical studies in cultured cells showed that HL, unlike LPL, was secreted by cld/cld hepatocytes, indicating the cld mutation probably affects LPL and HL in different ways. Sucrose gradient centrifugation studies (see below) showed LPL in cld/cld cells is an aggregate of monomers. That Mr of LPL subunits in cld/cld brown adipocytes is smaller than that in 3T3-L1 murine adipocytes treated with an inhibitor (CSTP) of ER glucosidase I (see below) suggests oligosaccharide chains of LPL may be synthesized in cld/cld cells with a subnormal number of sugar residues, or trimming of oligosaccharide chains may proceed at least beyond the action of glucosidase I. Whether the cld mutation affects primarily synthesis/processing of oligosaccharides of LPL in ER, transport of the lipase from ER to Golgi, or some other event, is to be resolved.

Blocking glucosidase I in ER with CSTP resulted in synthesis in 3T3-L1 adipocytes of endo H-sensitive lipase which was inactive and not secreted. Sucrose gradient centrifugation studies of LPL extracted from intracellular cisternae of brown adipocytes showed that endo H-sensitive LPL from CSTP-treated cells was an aggregate of inactive monomers, whereas endo H-sensitive lipase from cells in which Golgi mannosidase I or II had been blocked was dimerized. LPL in the latter cells was also active and secreted. Thus, processing high mannose-type chains to endo H-resistant chains is not required for dimerization, activity or secretion of LPL. The importance of processing LPL to endo H-resistance is not yet known.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15404-07 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructural Immunocytochemistry of Lipid Metabolism in Cells and Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	E. Joan Blanchette-Mackie	Research Biologist	LCDB, NIDDK
Others:	Robert O. Scow	Chief, Endocrinology Sect.	LCDB, NIDDK
	Nancy K. Dwyer	Biologist	LCDB, NIDDK
	Robert A. Coxey	IRTA, Post Doctoral Fellow	LCDB, NIDDK

COOPERATING UNITS *(if any)*

Dr. Peter Pentchev. Dev Metab. Neurol Branch, NINCDS, NIH; Dr. Howard Kruth Lab Exptl Ather, NHLBI, NIH; Dr. Constantine Londos, Lab Cell Develop Biol, NIDDK, NIH; Dr. Michael C. Schotz, Dept. of Medicine, U. California, CA.

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We located hepatic lipase in liver, both extravascularly and on the vascular endothelium with two antibodies to purified hepatic lipase. That two different antibodies identify hepatic lipase in two different sites in liver suggests each may be recognizing different isotopes of the lipase specific to each site. Combined lipase-deficient (cld/cld) mice have functional deficiencies of both lipoprotein lipase and hepatic lipase. We located hepatic lipase and lipoprotein lipase in cld/cld livers and cultured hepatocytes and found that hepatic lipase is present extracellularly while lipoprotein lipase accumulates within hepatocytes. It appears that the intracellular processing of these two non-functional glycoproteins is different in hepatocytes from cld/cld mice.

Niemann-Pick Type-C (NP-C) disease is a cholesterol storage disorder in which unesterified cholesterol derived from LDL accumulates intracellularly in lysosomes and Golgi. The relative distribution of LDL-derived cholesterol in Golgi compartments of normal and NP-C fibroblasts incubated with LDL for 24 hours was determined with freeze-fracture cytochemistry. Filipin complexes with cholesterol, forming characteristic deformations in Golgi membranes which can be quantitated. The highest density of membrane deformations in normal fibroblasts was present in Golgi condensing vacuoles and that in NP-C fibroblasts was in Golgi trans cisternae. Golgi condensing vacuoles of normal fibroblasts had more membrane deformations than those of NP-C fibroblasts indicating that translocation of cholesterol to condensing vacuoles is deficient in mutant cells. Impaired transport of cholesterol through Golgi to condensing vacuole membranes could explain the defective mobilization of cholesterol and transport to the plasma membrane in NP-C cells.

The cellular location of perilipin, an adipocyte specific phosphoprotein, was studied with electron microscopic immunocytochemistry in cultured 3T3-L1 adipocytes. Membrane leaflets of endoplasmic reticulum were continuous with the monolayer in contact with the lipid droplet core. Immunogold labeling for perilipin was found on the surface monolayer of the lipid droplets and also within cisternae of the surrounding endoplasmic reticulum. These results indicate that perilipin is located on the leaflet of endoplasmic reticulum surrounding lipid droplets.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15500-31LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Large-Scale Processing of Biological Material

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Joseph Shiloach	Research Chemist	LCDB:NIDDK
Others:	Jeanne B. Kaufman	Biologist	LCDB:NIDDK
	Shaul Reuveny	Guest Researcher	LCDB:NIDDK
	Yong-Jick Kim	Visiting Fellow	LCDB:NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER.
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Pilot Plant (Biotechnology Unit) combines several different types of activities. It is responsible for the large-scale production of bacteria, mammalian cells and biologically active compounds from various sources. Parallel to this activity, it conducts process development work associated with these preparations in order to be able to execute them efficiently. In addition, the unit carries on research work not necessarily associated with a current project, but work that has long-term implications for the Unit's performance.

During the last year, the Unit produced 185 different large-scale preparations, including micro-organism grown in volumes from 10 to 300 liters, mammalian cell growth up to 50 liter volumes and processing of various biological materials.

Process development work was done on large-scale purification of two proteins needed for the first phase of clinical trials. One is a modified form of *Pseudomonas aeruginosa* exotoxin A from recombinant *E. coli* and the second is S antigen from bovine retinas.

Research was conducted on developing an adaptive control strategy for maintaining the dissolved oxygen level in the aerobic growth of micro-organisms.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15503-10 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Developmental Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Alan R. Kimmel	Research Chemist	LCDB:NIDDK
Others:	John L. Medabalimi	Staff Fellow	LCDB:NIDDK
	Gail T. Ginsburg	Biologist	LCDB:NIDDK
	Rachel Gollop	Visiting Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

Drs. A. Greenberg and C. Londos, LCDB
Dr. Peter Devreotes, Johns Hopkins School of Medicine

LAB/BRANCH
Laboratory of Cellular & Developmental Biology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have used Dictyostelium and mammalian adipocytes as parallel systems to study the regulation of differentiation by extracellular signalling. Dictyostelium responds to secreted, extracellular cAMP which regulates development through receptor/G-protein signalling systems. We have fully characterized the cAMP receptor family with respect to gene organization, protein structure and spatial and temporal expression. Binding specificities for each receptor have been described and suggest that appropriate cAMP analogs will only activate a single cAMP receptor and its linked effector systems. DNA elements required for expression of each receptor have also been identified. These analyses permit a detailed study of receptor regulation and function through promoter fusion studies and expression of chimeric receptors in receptor null cells. 3T3-L1 cells will differentiate into adipocytes after exposure to dexamethasone. This hormone will also inhibit the synthesis of the lymphokine in these cells. We have shown that addition of IL-6 to 3T3-L1 cells treated with dexamthesone inhibits differentiation and the expression of adipocyte-specific gene expression. Other studies on adipocyte-specific gene expression are in progress. Perilipin is a protein associated with periphery of lipid droplets in adipocytes. We have isolated cDNAs for perilipin and deduced its amino acid sequence. We have shown that perilipin is expressed specifically in adipocytes and showed that its developmental regulation is associated with the appearance of lipid droplets but other adipocyte-specific genes have been used to study gene expression during differentiation of 3T3-L1 adipoblasts and in primary cultures of isolated adipocytes.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15505-14 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Adipocyte Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Constantine Londos	Research Chemist	LCDB:NIDDK
Others:	John J. Egan	Staff Fellow	LCDB:NIDDK
	Andrew S. Greenberg	Senior Staff Fellow	LCDB:NIDDK
	Janice L. Theodorakis	IRTA Fellow	LCDB:NIDDK
	Sheree A. Wek	Biologist	LCDB:NIDDK

COOPERATING UNITS *(if any)*

AR Kimmel, J Blanchette-Mackie, LCDB:NIDDK; RP Nordan, DCT:NCI; Christina Rondinone, LTPD:CH; P. Coon , AP Goldberg, U. Maryland, Baltimore

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Membrane Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Three areas of research on hormonal control of adipocyte metabolism are summarized: (A) Previously, we reported the discovery of perilipin, an adipocyte-specific protein that is associated with the lipid storage droplet and multiply phosphorylated upon elevation of cAMP. From an adipocyte expression library we have obtained cDNA that contains the entire predicted coding sequence for perilipin. Immunocytochemistry at the electron microscope level shows that the protein is associated intimately with the lipid surface in storage droplets. However, studies in biotin-deficient cells, in which lipid accumulation but not perilipin synthesis is depressed indicate that perilipin production is related to lipid surface area, rather than lipid mass. (B) Extending our finding that insulin rapidly activates a phospholipase activity and protein kinase C activity in adipocytes, we have found that adipocytes contain at least five of the known protein kinase C isozymes. Moreover, plasma membranes of cells stimulated with insulin, vasopressin, and phorbol ester reveal different enzyme profiles upon FPLC chromatography, indicating that these agents stimulate different protein kinase C isozymes. (C) Previously, we found that Interleukin-6 (IL-6) acts directly on adipocytes to inhibit lipoprotein lipase activity, data that suggest a role for this cytokine in cachexia. Also, endogenous IL-6 production by precursor 3T3-L1 fibroblasts was strongly inhibited by dexamethasone, an agent which stimulates the cells to differentiate into adipocytes. We now find that addition of exogenous IL-6 together with dexamethasone inhibits differentiation. Moreover, at all stages during the differentiation process, tumor necrosis factor stimulates IL-6 production. Taken together, these observations strongly suggest a pathophysiological autocrine role for IL-6 in adipocyte metabolism and as a mediator of other cytokines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15506-07 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Control of Gene Expression in Early Mammalian Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.:	Jurrien Dean	Section Chief	LCDB, NIDDK
Others:	Li-Fang Liang	Staff Fellow	LCDB, NIDDK
	Sarah Millar	Visiting Associate	LCDB, NIDDK
	Dwayne Lunsford	Staff Fellow	LCDB, NIDDK
	Eric Lader	IRTA Fellow	LCDB, NIDDK

COOPERATING UNITS *(if any)*

Frank Robey, LCDO, NIDR
Kenneth Tung, Washington University, St. Louis, MO
Stephen Beebe, Eastern Virginia Medical School

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Mammalian Developmental Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The mammalian zona pellucida is an extracellular matrix comprised of three sulfated glycoproteins (ZP1, ZP2 and ZP3) that surrounds growing oocytes, ovulated eggs and embryos. The zona proteins not only have vital roles in fertilization and early development, but the genes that encode them represent a paradigm for the investigation of mechanisms of oocyte-specific gene expression. We have previously described the genetic loci of mouse Zp-2, mouse Zp-3 and human ZP3 genes and have recently characterized the human ZP2 gene and a full-length cDNA of its transcript. Like the mouse and human ZP3 genes, the two ZP2 genes are well conserved: each is composed of 18 exons spanning approximately 12.5 kbp of DNA; the coding regions are 72% the same; and the resultant protein products are 60% identical. We have recently confirmed the presence of a second human ZP3 allele that results in a truncated protein product that is 52 amino acids shorter than the previously described allele. Both alleles are present in high frequency in human populations. Studies are currently underway to ascertain if the allele encoding the truncated protein has an adverse effect on human fertilization and early development.

The spatial and temporal expression of the mouse zona pellucida genes is precisely regulated. Zp-2 and Zp-3 transcripts are expressed only in oocytes where they accumulate to high abundance during a narrow two week period of oocyte growth. The 5' flanking regions of mouse Zp-2 and Zp-3 genes and their human homologs contain five short DNA sequences (4-12bp) that are 60-100% conserved and are approximately equidistant upstream of the TATAA box. Mutation of these five elements (I, IIA, IIB, III, IV) in ZP-luciferase constructs demonstrated that the 12bp element IV, positioned approximately 180 base pairs upstream from the TATAA box, is necessary and sufficient for high level expression from both mouse Zp-2 and Zp-3 promoters microinjected into the nucleus of 50 mu m diameter oocytes. Oligonucleotides containing the conserved element from either Zp-2 or Zp-3 form DNA-protein complexes of identical mobility in gel retardation assays using extracts from oocytes but not from other tissues. These data are consistent with the hypothesis that common factor(s) binding to the conserved element IV are involved in the coordinate expression of the oocyte-specific Zp-2 and Zp-3 zona pellucida genes.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK 15508-04 LCDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Chromatin Structure in Regulation of Mammalian Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.:	Ann Dean	Research Chemist	LCDB, NIDDK
Others:	Qi-Hui Gong	Visiting Scientist	LCDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

The transcription of eukaryotic genes encoding tissue and developmental stage specific proteins is a complex process involving the interaction of nuclear chromatin with RNA polymerase II, as well as with other transcriptional regulatory factors. The individual members of the globin gene family are temporally regulated to bring about the sequential expression of embryonic, fetal, and adult hemoglobins in the erythroid tissues of many species. The study of these genes has contributed much to our understanding of how selective transcription is accomplished. The epsilon-globin gene is the first of the beta-like globin genes to be expressed during human development. To investigate the regulation of this gene we have mapped, in vitro, the sites of interaction between nuclear proteins from erythroid and non-erythroid cells, and DNA sequences in the epsilon-globin promoter. We found that the erythroid factor GATA-1 bound to three sites in the promoter, and other factors, including SP-1, bound to the CACCC and CCAAT sites. Using a CAT reporter construct in transient expression assays, we found that the GATA-1 site at -165 is required for transcriptional enhancement by two erythroid specific enhancers. This site has been conserved during the evolution of the mammalian embryonic globin genes. The enhancers each contain their own complement of binding sites for (often the same) nuclear proteins emphasizing the modular nature of transcriptional regulatory regions. In each case the positive-acting regions of the enhancers consisted of AP-1 motifs. Thus, productive promoter-enhancer interactions increasing transcription of the eta-globin gene may require as few as two proteins interacting through two regulatory sites in the DNA.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY AND METABOLISM

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory conducts research in several apparently disparate areas that include morphogenesis, development, endocytosis, endocrinology, membrane transport, detoxication, and the physical and chemical behavior of proteins and nucleic acids. It does so by applying a broad array of different approaches. Resolution is being attempted by methods that stem from enzymology, biophysics, carbohydrate chemistry, cell biology and molecular biology. Although seemingly diverse, the common element to each of the research areas is appropriate to the Laboratories' designation: biochemical, metabolic and physical approaches are being brought to bear on major problems encompassed by the Institute's charge. It is the close proximity of experienced investigators from diverse scientific disciplines, able to discuss their individual problems with each other, that provide synergistic effects for resolution of the questions under investigation.

I. ENZYMES: FUNCTIONAL AND ABNORMAL

Several groups are active in this broadly designated area which covers the discovery of the several lesions in the gene for an enzyme whose absence leads to disease; the means by which enzymes are folded into the proper, active configuration, and the enzymatic mechanism by which imides are metabolized and detoxified.

Inherited Disorders of Lysosomal Function

One group is investigating several lysosomal enzymes, mutation of which may be responsible for certain metabolic diseases. The "protective protein" exists in a complex with the lysosomal enzymes beta-galactosidase and neuraminidase and is necessary for their activities. Its deficiency results in a lysosomal recessive disorder, galactosialidoses. Mice which are homozygous null mutants for the protective protein will be generated to aid in understanding the function of this protein and the deficiency disease. As a first step in this project, a plasmid has been constructed which is composed of 6.5 kilobases of mouse genomic protective protein DNA, inclusive of exons 1, 2 and 3, and a promoterless selectable marker, the neomycin resistance gene, inserted in frame after the protective protein ATG start site present in exon 2. The construct will now be used to effect targeted gene disruption of the protective protein locus in pluripotent embryonic stem cells by homologous recombination. The construct should enrich for selection of the rare homologous event since the promoterless neomycin resistance gene would remain transcriptionally silent after most non-homologous insertions. Reintroduction of stem cells, which have undergone homologous recombination into mouse blastocytes, passes the disrupted gene into the germline. Interbreeding of heterozyicgot siblings should yield animals homozygous for the disruption.

Mucolipidoses II and III are recessive genetic disorders caused by a deficiency of N-acetyl glucosaminyl-lysosomal enzyme-1-phosphotransferase, a membrane-bound enzyme pivotal in equipping lysosomal enzymes with phosphorylated mannose moieties, residues which target these enzymes to lysosomes. Since the phosphotransferase selects lysosomal enzymes as specific substrates by a conformationally dependent protein determinant shared by these enzymes, attempts are being made to purify the phosphotransferase by using its affinity to the recombinant lysosomal enzyme, beta-hexosaminidase B. With HeLa cell membranes as a source of enzyme, the phosphotransferase was bound in solution to beta-hexosaminidase B and the complex was isolated in very good yield with an anti-beta-hexosaminidase B antibody.

Enzymatic Basis of Detoxication

Among the few hydrolytic enzymes active in the detoxication process, are imidase and the carboxylesterases. Several cytosolic carboxylesterases from rat liver have now been purified to homogeneity and characterized, two of which appear to be responsible for the hydrolysis of aspirin.

Imidase, also a cytosolic protein, has been purified to homogeneity from rat liver. As with all of the enzymes of detoxication, imidase is active with a broad range of substrates that include such cyclic imides as hydantoins; succinimide, glutimide and adipimide; dihydrouracil and dihydrothymidine; and the aliphatic imide, deacetylimide. The hydrolysis of the two pyrimidines to their respective ureido derivatives requires magnesium ions; reaction with the two pyrimidines is experimentally reversible. For the hydrolysis of uracil to yield N-carbamyl-beta-alanine, the equilibrium constant was found to be 1 at pH 5.4. The enzyme exhibits stereoselectivity for one of the isomers of 2-methylhydantoin and stereospecificity for one of the isomers of 2-isopropylhydantoin and of 5-phenylsuccinimide. Imidase is a protein of about 240,000 daltons composed of four subunits of equal size. It is thought to catalyze a reaction in the degradative pathway of uridine and thymidine under the label of dihydropyrimidinase or hydropyrimidine hydrase; the term imidase reflects the broader potential of the enzyme that has now been purified.

Protein Structure and Enzyme Mechanisms

One laboratory is engaged in studies on protein structure and the mechanism by which the enzyme molecule, synthesized as a random coil, can fold into a specific secondary and tertiary structure without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of its first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. Interest is in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. By rapid kinetic techniques, e.g. stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

II. MORPHOGENESIS AND DEVELOPMENT

At a higher level of organization, one can question how enzymes or regulatory genes affect cell structures or the function of entire glands.

Polysaccharides in Morphogenesis

Enzymatic determinations in Calcofluor-resistant (cal1) mutants of Saccharomyces cerevisiae as well as cloning and sequencing of the CAL1 gene indicated that these mutants lack the newly discovered chitin synthetase 3 (Chs3). Examination of strains lacking either chitin synthetase 2 (Chs2) or Chs3 by electron microscopy and by specific staining of chitin, indicated that Chs3 is responsible for the chitin ring formed at the base of emerging buds and for chitin dispersed in the cell wall, whereas Chs2 is required for the chitin disk synthesized between mother and daughter cells at cytokinesis. Chs3 is also involved in the diffuse deposition of chitin in cell cycle mutants incubated at a nonpermissive temperature. Finally, Chs3 is required for the formation of a chitosan layer in spore walls. Because simultaneous deficiencies in Chs2 and Chs3 are lethal, it is suggested that inhibitors of both Chs2 and Chs3 may be effective antifungal agents.

Tissue Specific and Hormone Regulated Gene Expression

The molecular basis of mammary-specific and developmentally and hormonally regulated gene expression is being studied through analyses of the mouse whey acidic protein (WAP) gene in transgenic mice. The mammary specific elements were shown to be located within the promoter region, and the absence of expression in early pregnancy was found to be due to a repressor which keeps the gene inaccessible to transcription factors produced throughout pregnancy. WAP gene expression during pregnancy and lactation is predominantly regulated on a transcriptional rather than a post-transcriptional level. Continuing attempts to establish the mammary gland as a bioreactor, the mouse WAP gene was expressed in transgenic pigs. Expression levels of the mouse WAP gene in pigs were higher than in mice, and a more accurate regulation was observed. WAP shows strong structural similarities with protease inhibitors, suggesting that it may have a function in addition to being a caloric nutrient. Female mice from about 25% of the lines and about 50% of the transgenic pigs failed to produce milk after parturition and exhibited a *milchlos* (agalactic) phenotype. Alveolar development and histological appearance of mammary tissue from post-partum *milchlos* mice were similar to that of glands from non-transgenic mice at mid-pregnancy. Precocious expression of WAP during pregnancy was associated with a non-functional mammary gland suggesting that WAP may play a role in mammary development.

III. PROTEIN SORTING AND TRANSPORT FUNCTIONS

Central to modern biology is the nature of the mechanism for the movement of macromolecules, glycoproteins in particular, not only into and out of the cell but also into specific organelles. Plural mechanisms are being sought from the viewpoint of the disciplines of somatic cell genetics, molecular biology, carbohydrate chemistry, endocrinology and biochemistry. The implications of the work extend from cell biology to applications in thyroid pathobiology and approaches to the AIDS virus.

Role of the Carbohydrate Moiety of Glycoproteins in Cellular Activity

Earlier studies that had been designed to clarify the functional role of the hepatic receptor for asialoglycoproteins and to establish the biochemical mechanisms involved in receptor modulation, were continued. Using a human hepatoblastoma cell line, HepG2, as a model system, it was found that incubation of these cells in the presence of 10 mM N-acetyl-D-mannosamine markedly increased the normal complement of cell surface sialic acid with resultant up modulation in the activity of the asialoglycoprotein receptor. A second and unexpected result was the threefold stimulation in fucose incorporation, indicative of a complex metabolic interaction between these two inversely related sugars. Thirdly, an examination of the role of cGMP as second messenger revealed that it restored normal ligand binding but was unable to produce the hyperbinding activity that results from high levels of glucose; cAMP blocked the effect of cGMP. The significance of the above findings relates to analogous changes reported in the livers of diabetic rats.

Role of the Nuclear Envelope in Intracellular Protein Sorting

Macromolecular transport across the nuclear pore complex, essential for the regulation of cell growth and development, is under investigation. Proteins bearing cytoplasmically oriented, O-linked GlcNAc were shown to be components of the nuclear pore complex. An *in vitro* binding and nuclear import assay using radiochemically or fluorescently labelled conjugates was used to study nuclear transport. Conjugates having the proper sequence bind to nuclei with a Kd of 100nM. The nuclear membrane is a self assembling structure; when DNA was added to extracts of eggs of *Xenopus laevis*, nuclei reformed; labelled conjugates entered the reformed nuclei. To examine the function of nuclear pore glycoproteins in nuclear transport, extracts were depleted of O-linked GlcNAc glycoproteins using wheat germ agglutinin affinity chromatography. Nuclei formed from the depleted extracts did not import proteins unless O-linked GlcNAc glycoproteins were added back to the extracts. The rat gene encoding p62 is devoid of introns, has two potential transcription start sites and bears a functional 5' promoter-enhancer region. p62 has three domains: one myosin-like one Ser/Thr rich and one collagen-like; after transfection, the protein was overexpressed in heterologous cells and a number of deletion mutants were constructed. Using the cloned cDNA, O-linked GlcNAc addition was shown to be catalyzed *in vitro* in a rabbit reticulocyte lysate at sites previously mapped. The expressed protein serves as a substrate for the O-linked GlcNAc transferase. The availability of recombinant p62 and deletion mutants should allow a functional dissection of the domains of this essential nuclear pore component.

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

Results of inhibitor studies indicate that microtubules play an important role in post-internalization steps of the endocytic pathway. One group's approach to dissecting the process of endocytosis is the isolation and analysis of mutant cells. The Chinese hamster ovary cell mutant FD1-3-25 manifests marked alterations in the appearance of both its microtubules and late endosomes. Aberrant association of these structures is observed in the mutant under non-physiological conditions that exaggerate microtubule-dependent movement of late endosomes. Taxol-dependent polymerization shows a 35 kDa polypeptide persistently associated with microtubules of the mutant; this polypeptide is only loosely associated with microtubules from parental cells. Studies directed toward identification of the polypeptide and determination of its role in endocytosis are in progress.

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

Specialized features of iodine metabolism in thyroid include iodide transport across the basal membrane, iodide efflux into the follicular lumen, and iodination of thyroglobulin. The process is regulated by TSH and other hormones, as well as by iodine. These studies are integral to understanding thyroid hormone synthesis. Signal systems important in regulation of iodide transport and metabolism were developed over several years and used thyroid cells in culture. Iodide transport was found to be regulated by cAMP and calcium/phospholipid pathways. Further studies of the iodide transport in FRTL-5 cells confirmed and extended work that characterized iodide transport in intact tissues. Stilbenes, agents that interact with band 3, also specifically effected the transport of iodide by the thyroid. Band 3, the major integral membrane protein of mammalian erythrocytes is specifically effected by stilbenes. Band 3 functions to exchange internal bicarbonate for external chloride. Studying this function in the laboratory and using a cDNA probe to murine band 3, several cDNA clones were isolated using expression libraries made from FRTL-5 cells. Using the clones to transform cells that normally do not transport iodide, present studies transfect FRTL-5 clones into COS-7 cells to address their role in iodide transport, a role previously suggested by studies showing effects of stilbenes on iodide transport in the thyroid. Recent studies of goiter show that in addition to low-iodinated thyroglobulin, albumin and hemoglobin are prominent proteins in goiter tissues. The thyroid, as a site of synthesis of serum-proteins, has been addressed using mRNA from goiter tissues. Goiter from one patient with congenital goiter unexpectedly showed the absence of the sialyltransferase activity that is necessary to sialylate thyroglobulin.

Cell Regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes

This laboratory is studying the structure-function relationships of the thyrotropin (TSH) receptor, the involvement of this receptor in autoimmune Graves' disease, including its connective tissue complications (exophthalmos and pretibial myxedema), as well as the relationship between thyroid autoimmune diseases and other organ-specific autoimmune diseases. Structure/function comparisons to other glycoprotein hormone receptors are being examined and the interdependent regulation of thyroid function and growth by the TSH receptor and other receptors is being evaluated. Particular attention was given to identifying determinants on the receptors that are important for ligand interactions and signal transduction as well as transcriptional and post-transcriptional mechanisms by which TSH and the other receptors affect gene expression. The relationship between oncogene transformation, and loss of normal receptor regulation of thyroid function and growth, was found to be linked to the development of thyroid tumors and adenomas. A number of other topics are under investigation: the role of different signal transduction mechanisms in thyroid cell growth and differentiation; the role of phosphate and carbohydrate moieties in thyroglobulin structure and post-translational processing; and the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis. The role of the major histocompatibility antigens in the development of autoimmune thyroid diseases and organ specific immune diseases are being studied. A long-term continuing project involves the development of thyroid cells which can grow in continuous culture in vitro and act as models of endocrine and thyroid disorders.

The Role of Intracellular Traffic in HIV Infection

The REV protein of HIV was shown to lead to the premature transport of the partially spliced mRNA, encoding the envelope glycoprotein, from the nucleus to the cytoplasm. To develop a system for examining the effects of REV, a means was devised for generating nuclei *in vitro* around exogously added DNA. This method involves using extracts from *Xenopus laevis* where it has been demonstrated that nuclear assembly can be reconstituted. These reconstituted nuclei are capable of transporting proteins to the nuclear interior. The resultant preparations allowed examination of the mechanism of REV nuclear transport and function. In other studies, examination has begun of the structure of the nuclear pore complex, across which REV, TAT, and other viral proteins cross after viral infection. The major nuclear pore glycoprotein p62 has been cloned, sequenced and expressed in cultured cells and in *E. coli*. The gene encoding this protein was isolated and found to be 2.95 kb in length and devoid of introns. The promoter-enhancer region was identified and constructs were made with which expression of the p62 gene can be monitored. The movement of nucleic acids across the nucleus is also being investigated.

IV. HYDRATION FORCES

The more physical interactions of macromolecules - particularly proteins, lipids and DNA - with their environment is being studied with special focus on the role of water.

Direct Measurement of Forces between Membranes or Macromolecules

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular assembly and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, DNA double helices, and stiff linear polysaccharides. During the current year, energy, entropy and enthalpy of water release from hydrated ordered arrays of DNA molecules into a bathing medium has been measured. It has been shown that the negative entropy of structured water in the ordered DNA phase is an exponentially decaying function of interhelical separation with approximately a 4 Angstrom decay length. Entropically favored assembly is a common feature of many biologically important processes.

A theory has been developed explaining spontaneous dehydration transitions in multilayer lipid systems followed by the formation of tight molecular contacts between the membranes. It has been shown that lateral correlation in the positions of solvated surface groups is the main factor determining the point of the transition. A new model for the interaction of neutral phospholipid membranes has been suggested that includes constraints on the motion of surface charged groups imposed by the bilayer structure.

Physics of Ionic Channels and other Proteins with Aqueous Cavities

Through the use of osmotic stress, some 60 water molecules were found to bind to hemoglobin when it switches from the "tense" deoxy to the "relaxed" oxy forms. It appears that this water should be treated as a liganding species much as oxygen or other allosteric effectors. The alpha-toxin trans-membrane channel from Staphylococcus aureus has been reconstituted into bilayer membranes and osmotically stressed to provide the information that 600 water molecules enter and leave this channel when it opens and closes under applied voltage. This channel appears also to have different pH sensitivity from each side of the membrane. Swelling of secretory granules after release from beige mouse mast cells appears to be governed by hydration/dehydration events that can be described by thermodynamic analysis similar to that developed to analyze macromolecular assembly in vitro. The coupling of swelling concomitant to secretion with the exchange of counter ions to the polysaccharide matrix suggests a further parallel between in vivo and in vitro processes.

Structure and Physical Properties of DNA and DNA-Protein Complexes

The DNA sequences responsible for the large, Zn induced bend within TF IIIA binding domain of the 5s RNA gene from Xenopus, seen previously, have been more closely identified. Phased junctions between natural B-form DNA and oligopurine tracts with an altered, Zn dependent conformation seem the most probable cause of the observed bend. There is observed correlation between binding strength of the gene regulatory protein TF IIIA to sequence variants of the 5s RNA gene and the Zn induced bending magnitude of these DNA fragments. The observed bending of the DNA by TF IIIA binding is very probably accomplished by stabilizing the same DNA conformation as Zn binding.

A link between the increased flexibility of the LMM-HMM hinge in assembled minifilaments with carboxy terminal phosphorylation of Acanthamoeba myosin II and actin activated ATPase activity has been uncovered. The same, very distinctive dependence of ATPase activity of copolymeric minifilaments on phosphorylated myosin fraction is also observed in the flexibility of the HMM arms. A fairly stiff spring constant for distortion of the HMM arm attached to the actin appears necessary for myosin function.

The osmotic sensitivity of the B to Z form transition of poly(dG-mdC) has been measured. About 2 water molecules per base pair are released. The interplay of salt concentration and osmotic stress in inducing the transition shows that molecular hydration, not electrostatics, plays the dominant role in determining DNA structure energetics.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 17001-25 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
The Role of the Carbohydrate Moiety of Glycoproteins in Cellular Activity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Ashwell	Institute Scholar	LBM, NIDDK
Others:	W. Berlin	IRTA	LBM, NIDDK
	O. Gabriel	Research Chemist	LBM, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.75	1.75	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The work carried out during the course of this year represents a continuation of earlier studies designed to clarify the functional role of the hepatic receptor for asialoglycoproteins and to establish the biochemical mechanisms involved in receptor modulation. Using the human hepatoblastoma cell line HepG2 as a model system, it was found that incubation of these cells in the presence of 10 mM N-Acetyl-D-mannosamine markedly increased the normal complement of cell surface sialic acid with resultant up modulation in the activity of the asialoglycoprotein receptor. A second and unexpected result was the threefold stimulation in fucose incorporation, indicative of a complex metabolic interaction between these two inversely related sugars. Thirdly, an examination of the role of cGMP, as second messenger, revealed that it restored normal ligand binding but was unable to produce the hyperbinding activity produced by high levels of glucose; cAMP blocked the effect of cGMP. The significance of the above findings relates to analogous changes reported in the livers of diabetic rats.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17002-21 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Enzymatic Basis of Detoxication

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	W.B. Jakoby	Chief, LBM	LBM, NIDDK
Others:	Y-S. Yang	Visiting Fellow	LBM, NIDDK
	X. Chen	Visiting Fellow	LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

Among the few hydrolytic enzymes active in the detoxication process, are imidase and the carboxylesterases and imidase. Several cytosolic carboxylesterases from rat liver have now been purified to homogeneity and characterized, two of which appear to be responsible for the hydrolysis of aspirin.

Imidase, also a cytosolic protein, has now been purified to homogeneity from rat liver. As with all of the enzymes of detoxication, imidase is active with a broad range of substrates that include such cyclic imides as hydantoins; succinimide, glutimide and adipimide; dihydrouracil and dihydrothymidine; and the aliphatic imide, deacetylimide. Only the hydrolysis of the two pyrimidines to their respective ureido derivatives requires magnesium ions; with these two there is an observable reverse reaction. For the hydrolysis of uracil to yield N-carbamyl-beta-alanine, the equilibrium contrast was found to be 1 at pH 5.4. The enzyme exhibits stereoselectivity for one of the isomers of 2-methylhydantoin and stereospecificity for one of the isomers of 2-isopropylhydantoin and of 5-phenylsuccinimide.

Imidase is a protein of about 240,000 daltons composed of four subunits of equal size. It is thought to catalyze one of the reactions in the degradative pathway of uridine and thymidine under the label of dihydropyrimidinase and hydropyrimidine hydrase; the term imidase reflects the broader potential of the enzyme that has now been purified.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17003-24 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Polysaccharides in Morphogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Cabib	Senior Research Chemist	LBM, NIDDK
Others:	P.C. Mol	Visiting Fellow	LBM, NIDDK
	J.A. Shaw	IRTA	LBM, NIDDK
	W.-J. Choi	Visiting Fellow	LBM, NIDDK

COOPERATING UNITS *(if any)*

LCB, NHLBI (Blair Bowers); Departamento de Microbiologia, University of Salamanca, Spain (Angel Durán)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Developmental Biology

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
4	4	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Enzymatic determinations in Calcofluor-resistant (cal1) mutants of Saccharomyces cerevisiae as well as cloning and sequencing of the CAL1 gene indicated that those mutants lack the newly discovered chitin synthetase 3 (Chs3). Examination of strains lacking either chitin synthetase 2 (Chs2) or Chs3 by electron microscopy and specific staining of chitin indicated that Chs3 is responsible for the chitin ring formed at the base of emerging buds and for chitin dispersed in the cell wall, whereas Chs2 is required for the chitin disk synthesized between mother and daughter cells at cytokinesis. Chs3 is also involved in the diffuse deposition of chitin in cell cycle mutants incubated at a nonpermissive temperature. Finally, Chs3 is required for the formation of a chitosan layer in spore walls.

Simultaneous deficiency in Chs2 and Chs3 is lethal, therefore an inhibitor of both Chs2 and Chs3 may be an effective antifungal agent.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-9

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 17004-23 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. McPhie	Research Chemist	LBM, NIDDK

COOPERATING UNITS *(if any)*
LMC, NHLI (Robert Adelstein); LCP, NIDDK (Edith Miles); MB, NCI (Jane Cheng); LPS, DCRT (Richard Shrager)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1	1	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

This laboratory is engaged in studies on protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight= 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of its first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. Interest is in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction , their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 935

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17008-08 LBM

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of the Nuclear Envelope in Intracellular Protein Sorting

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.A. Hanover	Research Chemist	LBM, NIDDK
Others:	C. Starr	Guest Researcher	LBM, NIDDK
	M. Baker	Expert	LBM, NIDDK
	S. Bailer	Guest Researcher	LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Macromolecular transport across the nuclear pore complex is essential for the regulation of cell growth and development. The mechanism of nuclear transport is under investigation. The laboratory has demonstrated that proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. An in vitro binding and nuclear import assay using radiochemically or fluorescently labelled conjugates has been employed to study nuclear transport. Conjugates having the proper sequence bind to nuclei with a Kd=100nM. The nuclear membrane is a self assembling structure; when DNA is added to extracts of eggs of Xenopus laevis, nuclei reform. The labelled conjugates enter these reformed nuclei. To examine the function of nuclear pore glycoproteins in nuclear transport, extracts have been depleted of O-linked GlcNAc glycoproteins using wheat germ agglutinin affinity chromatography. Nuclei formed from the depleted extracts do not import proteins. When O-linked GlcNAc glycoproteins are added back to the extracts, the reformed nuclei are transport competent. The cDNA and gene encoding the major rat pore glycoprotein p62 have been characterized. The gene encoding p62 is devoid of introns, has two potential transcription start sites and a functional 5' promoter-enhancer region. p62 has three domains: one myosin-like one Ser/Thr rich and one collagen-like. By transfecting an expression construct encoding p62 the protein was overexpressed in heterologous cells. Deletion mutants expressing various domains of the protein have also been constructed. Using the cloned CDNA O-linked GlcNAc addition was shown to be catalyzed in vitro in a rabbit reticulocyte lysate. The deletion mutants have allowed mapping of the sites of O-linked GlcNAc addition. p62 has been expressed in *E. coli* and produced in milligram quantities. The protein serves as a substrate for the O-linked GlcNAc transferase. The availability of recombinant p62 and deletion mutants should allow a functional dissection of the domains of this essential nuclear pore component.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-579

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17009-06 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Tissue Specific and Hormone Regulated Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	L. Hennighausen	Research Chemist	LBM, NIDDK
Others:	R.A. McKnight	IRTA	LBM, NIDDK
	A. Shamay	Visiting Fellow	LBM, NIDDK

COOPERATING UNITS (if any)
US Department of Agriculture (Robert Wall, Vernon Pursel)

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The molecular basis of mammary-specific and developmentally and hormonally regulated gene expression is being studied through analyses of the mouse whey acidic protein (WAP) gene in transgenic mice. It was shown that mammary specific elements are located within the promoter region, and that the absence of expression in early pregnancy is due to a repressor which keeps the gene inaccessible to transcription factors produced throughout pregnancy. WAP gene expression during pregnancy and lactation is predominantly regulated on a transcriptional rather than a post-transcriptional level.

In continuation of establishing the mammary gland as a bioreactor, the mouse WAP gene was expressed in transgenic pigs. Expression levels of the mouse WAP gene in pigs were higher than in mice and a more accurate regulation was observed.

WAP shows strong similarities with protease inhibitors, suggesting that it may have a function in addition to being a caloric nutrient. Female mice from about 25% of the lines and about 50% of the transgenic pigs failed to produce milk after parturition and exhibited a *milchlos* (agalactic) phenotype. Alveolar development and histological appearance of mammary tissue from post-partum *milchlos* mice were similar to that of glands from non-transgenic mice at mid-pregnancy. Precocious expression of WAP during pregnancy was associated with a non-functional mammary gland suggesting that WAP may play a role in mammary development.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 17024-08 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Inherited Disorders of Lysosomal Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. Myerowitz	Research Chemist	LBM, NIDDK
Others:	J.A. Boose	Staff Fellow	LBM, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The "protective protein" exists in a complex with the lysosomal enzymes beta-galactosidase and neuraminidase and is necessary for their activities. Its deficiency results in a lysosomal recessive disorder, galactosialidoses. Mice which are homozygous null mutants for the protective protein will be generated to aid in understanding the function of this protein and the deficiency disease . As a first step in this project, a plasmid has been constructed which is composed of 6.5 kilobases of mouse genomic protective protein DNA inclusive of exons 1, 2 and 3 and a promoterless selectable marker, the neomycin resistance gene, inserted in frame after the protective protein ATG start site present in exon 2. The construct will now be used to effect targeted gene disruption of the protective protein locus in pluripotent embryonic (ES) stem cells by homologous recombination. The construct should enrich for selection of the rare homologous events since the promoterless neomycin resistance gene would remain transcriptionally silent after most non-homologous insertions. Reintroduction of stem cells, which have undergone homologous recombination into mouse blastocytes, passes the disrupted gene into the germline. Interbreeding of heterozygote siblings yields animals homozygous for the disruption.

Mucolipidoses II and III are recessive genetic disorders caused by a deficiency of N-acetyl glucosaminyl-lysosomal enzyme-1-phosphotransferase, a membrane bound enzyme pivotal in equipping lysosomal enzymes with phosphorylated mannose moieties, residues which target these enzymes to lysosomes. Since the phosphotransferase selects lysosomal enzymes as specific substrates by a conformationally dependent protein determinant shared by these enzymes, attempts are being made to purify the phosphotransferase via its affinity to the recombinant lysosomal enzyme, beta-hexosaminidase B. Utilizing Hela cell membranes as a source of enzyme, the phosphotransferase was bound in solution to beta-hexosaminidase B and the complex was isolated with an anti-beta-hexosaminidase B antibody. Seventy percent of the total activity was recovered in this manner.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 18007-12 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.F. Grollman	Medical Officer (Research)	LBM, NIDDK
Others:	M.C. Baggio	Visiting Fellow	LBM, NIDDK

COOPERATING UNITS *(if any)*
LBM, NIDDK (L.D. Kohn, G. Ashwell); Smithsonian Institute (R.J. Montali); DBB, OBR (N.Y. Nguyen); Univ. Sao Paulo (Medeiros-Neto).

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Laboratory of Biochemistry and Metabolism

SECTION
Section on Cell Regulation

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	2.00	0.25

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Specialized features of iodine metabolism in thyroid include iodide transport across the basal membrane, iodide efflux into the follicular lumen, and iodination of thyroglobulin. The process is regulated by TSH and other hormones, as well as by iodine. These studies are integral part of understanding thyroid hormone synthesis.

Signal systems important in regulation of iodide transport and metabolism were developed over several years and used thyroid cells in culture. These studies show that iodide transport is regulated by cAMP and calcium/phospholipid pathways. Further studies of the iodide transport in FRTL-5 cells confirmed and extended studies that characterized iodide transport in intact tissues. Stilbenes, agents that interact with band 3, also specifically effected the transport of iodide by the thyroid. Band 3, the major integral membrane protein of mammalian erythrocytes is specifically effected by stilbenes. Band 3 functions to exchange internal bicarbonate for external chloride. Studying this function in the laboratory and using a cDNA probe to murine band 3, several cDNA clones were isolated using expression libraries made from FRTL-5 cells. Using the clones to transform cells that normally do not transport iodide, present studies transfect FRTL-5 clones into COS-7 cells to address their role in iodide transport, a role previously suggested by studies showing effects of stilbenes on iodide transport in the thyroid.

Studies of goiter are completed. These studies show that in addition to low-iodinated thyroglobulin, albumin and hemoglobin are prominent proteins found in goiter tissues. The thyroid, as a site of synthesis of serum-proteins, has been addressed using mRNA from goiter tissues. Goiter from a patient with congenital goiter had an unexpected finding showing an absence of sialyltransferase activity necessary to sialylate thyroglobulin. This project has opened up a new opportunity to address tissue specific glycosyltransferase activity and function, and its potential hormonal regulation in the thyroid.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18008-25 LBM

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.D. Kohn	Medical Director, USPHS, and Chief, Sec on Cell Regulation	LBM, NIDDK
Others:	T. Ban	Visiting Fellow	LBM, NIDDK
	S. Ikuyama	Visiting Fellow	LBM, NIDDK
	S. Kosugi	Visiting Fellow	LBM, NIDDK
	M. Saji	Visiting Fellow	LBM, NIDDK
	K. Tahara	Special Volunteer	LBM, NIDDK
	H. Niller	Special Volunteer	LBM, NIDDK

COOPERATING UNITS *(if any)* NIDDK (E.F. Grollman); U. Pisa, Italy (A. Pinchera); U. Naples (R. DiLauro, E. Avvedimento, S. Aloj, & E. Consiglio); NIAID (C. Kozak); NCI (D. Singer); CCHD (W. Gahl); U. Texas Galveston (B. Prabhakar); U. MD (W.A. Valente); Tokyo Women's U, Tokyo, Japan (O. Isozaki); U. of Kyoto, Kyoto, Japan (T. Mori).

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SECTION

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER.
6.8	5.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This laboratory is studying the structure-function relationships of the thyrotropin (TSH) receptor, the involvement of this receptor in autoimmune Graves' disease, including its connective tissue complications (exophthalmos and pretibial myxedema), as well as the relationship between thyroid autoimmune diseases and other organ-specific autoimmune diseases, i.e. Lupus or diabetes. We examine structure/function comparisons to other glycoprotein hormone receptors and evaluate the interdependent regulation of thyroid function and growth by the TSH receptor and receptors for alpha 1-adrenergic agents, cholinergic agents, insulin, insulin-like growth factors (I and II), hydrocortisone, thyroid hormones, bacterial toxins (cholera and pertussis, for example), interferon, and interleukins. Particular attention is being given to identifying determinants on the receptors important for ligand interactions and signal transduction mechanisms as well as transcriptional and posttranscriptional mechanisms by which TSH and the other receptors affect gene expression. The relationship between oncogene transformation and loss of normal receptor regulation of thyroid function and growth is linked to the development of thyroid tumors and adenomas. We relate the role of different signal transduction mechanisms - cAMP, Ca/phosphoinositide and arachidonate - in thyroid cell growth and differentiation; the transport of T3, T4, monoiodotyrosine, diiodotyrosine, or other amino acids from the lysosome; the role of phosphate and carbohydrate moieties in thyroglobulin structure and post-translational processing; and the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis. We study the role of major histocompatibility antigens in the development of autoimmune thyroid diseases and organ specific immune diseases. These studies combine a molecular biology, cell biology and monoclonal antibody approach. A long term continuing project involves the development of thyroid cells which can grow in continuous culture in vitro and act as models of endocrine and thyroid disorders.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18009-12 LBM

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A.R. Robbins	Research Geneticist	LBM, NIDDK
Others:	C.W. Hall	Research Chemist	LBM, NIDDK
	T.M. Weber	Staff Fellow	LBM, NIDDK

COOPERATING UNITS *(if any)*

MDB, NIDDK, NIH (Dr. Karen MacKay); LI, NIDR, NIH (Dr. Constance Oliver)

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Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Results of inhibitor studies indicate that microtubules play an important role in post-internalization steps of the endocytic pathway. Our laboratory's approach to dissecting the process of endocytosis is the isolation and analysis of mutant cells. The Chinese hamster ovary cell mutant FD1-3-25 manifests marked alterations in the appearance of both its microtubules and late endosomes. Aberrant association of these structures is observed in the mutant under non-physiological conditions that exaggerate microtubule-dependent movement of late endosomes. Taxol-dependent polymerization shows a 35kDa polypeptide persistently associated with microtubules of the mutant; this polypeptide is only loosely associated with microtubules from parental cells. Studies directed toward identification of this polypeptide and determination of its role in endocytosis are in progress.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18010-04 LBM

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of Intracellular Traffic in HIV Infection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.A. Hanover	Research Chemist	LBM, NIDDK
Others:	M. Miller	IRTA	LBM, NIDDK

COOPERATING UNITS *(if any)*

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The REV protein of HIV has been recently shown to lead to the premature transport of the partially spliced mRNA encoding the envelope glycoprotein from the nucleus to the cytoplasm. To develop a system to examine the effects of REV, a means has been devised for generating nuclei in vitro around exogously added DNA. This method involves using extracts from Xenopus laevis where it has been demonstrated that nuclear assembly can be reconstituted. These reconstituted nuclei are capable of transporting proteins to the nuclear interior. These preparations allow examination of the mechanism of REV nuclear transport and function. In other studies, examination has begun of the structure of the nuclear pore complex, across which REV, TAT, and other viral proteins cross following viral infection. The structure of the pore complex is being explored using recombinant DNA techniques. The major nuclear pore glycoprotein p52 has been cloned, sequenced and expressed in cultured cells and in E. coli. The gene encoding this protein has been isolated and found to be 2.95 kb in length and devoid of introns. The promoter-enhancer region has been identified and constructs have been made with which expression of the p62 gene can be monitored. The availability of the primary sequence of this protein has allowed the preparation of antisera which react with p62. These antibodies are being screened for their ability to interfere nuclear transport. The movement of nucleic acids across the nucleus is also being investigated. In situ hybridization is being used to follow mRNA transport. In addition, the movement of double stranded DNA into the nucleus is monitored using fluorescence and luminometry. Understanding how retroviral products cross the nuclear envelope is critical to attempts to regulate or inhibit the critical steps in the HIV life cycle.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 18011-03 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Cell Specific Activity of Elements within the HIV-LTR

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Hennighausen	Research Chemist	LBM, NIDDK

COOPERATING UNITS *(if any)*

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SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was terminated as of September 30, 1990.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 18012-07 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Direct Measurement of Forces between Membranes or Macromolecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V.A. Parsegian	Guest Researcher	LBM, NIDDK
	D.C. Rau	Research Chemist	LBM, NIDDK
Others:	K. Gawrisch	Guest Researcher	LBM, NIDDK
	S. Leikin	Guest Researcher	LBM, NIDDK

COOPERATING UNITS *(if any)*
Brock University, Ontario (Dr. R.P. Rand); University of British Columbia, Vancouver, Canada (Dr. E.A. Evans); University of Minnesota, Minneapolis, MN (Dr. D.F. Evans); Tulane University, New Orleans, LA (Dr. W. Reed); Naval Research Laboratory (Dr. W. O'Grady)

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SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
2.3	2.3	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular assembly and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, DNA double helices, and stiff linear polysaccharides.

During the current year energy, entropy and enthalpy of water release from hydrated ordered arrays of DNA molecules into a bathing medium has been measured. It has been shown that the negative entropy of structured water in the ordered DNA phase is an exponentially decaying function of interhelical separation with approximately a 4 Angstrom decay length. Entropically favored assembly is a common feature of many biologically important processes.

A theory has been developed explaining spontaneous dehydration transitions in multilayer lipid systems followed by the formation of tight molecular contacts between the membranes. It has been shown that lateral correlation in the positions of solvated surface groups is the main factor determining the point of the transition.

A new model for the interaction of neutral phospholipid membranes that includes constraints on the motion of surface charged groups imposed by the bilayer structure has been suggested.

A system has been designed to produce macroscopic samples of oriented DNA for combined x-ray and NMR experiments to probe further water structuring.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18013-04 LBM

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Physics of Ionic Channels and other Proteins with Aqueous Cavities

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	V.A. Parsegian	Guest Researcher	LBM, NIDDK
Others:	S. Bezrukov	Special Volunteer	LBM, NIDDK
	M.F. Colombo	Visiting Fellow	LBM, NIDDK
	M.J. Curran	Special Volunteer	LBM, NIDDK
	J.J. Kasianowicz	IRTA	LBM, NIDDK
	C.R. Moore	IRTA	LBM, NIDDK
	D.C. Rau	Research Chemist	LBM, NIDDK

COOPERATING UNITS *(if any)* LPTB, NICHD (Dr. J.J. Zimmerberg); John Hopkins University, Baltimore, MD (Dr. A. Harris); Office of Naval Research (Dr. I. Vodyanoy); St. George's Hospital, University of London, England (Drs. C. Pasternak and L. Bashford); Worcester Foundation for Experimental Biology, Shrewsbury, MA (Dr. H. Bayley)

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SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
5.75	5.75	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Through the use of osmotic stress, it has been found that some 60 water molecules bind to hemoglobin when it switches from the "tense" deoxy to the "relaxed" oxy forms. It appears that this water should be treated as a liganding species much as oxygen or other allosteric effectors.

The alpha-toxin trans-membrane channel from Staphylococcus aureus has been reconstituted into bilayer membranes and osmotically stressed to provide the information that 600 water molecules enter and leave this channel when it opens and closes under applied voltage. This channel appears also to have different pH sensitivity from each side of the membrane.

Swelling of secretory granules after release from beige mouse mast cells appears to be governed by hydration/dehydration events that can be described by thermodynamic analysis similar to that developed to analyze macromolecular assembly in vitro. The coupling of swelling concomitant to secretion with the exchange of counterions to the polysaccharide matrix suggests a further parallel between in vivo and in vitro processes.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18014-07 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Structure and Physical Properties of DNA and DNA-Protein Complexes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: D.C. Rau Research Chemist LBM, NIDDK

COOPERATING UNITS *(if any)* LCB, NHLBI (Drs. E.D. Korn, C.L. Ganguly); LMB, NIDDK (Dr. J. Nickol); LCP, NIDDK (Dr. E. Charney); George Mason University, Fairfax, VA (Dr. H.-H. Chen); University of Nevada, Reno, Nevada (Dr. R.H. Harrington); University of Calgary, Alberta, Canada (Dr. D. Bazett-Jones)

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Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: .5

PROFESSIONAL: .5

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The DNA sequences responsible for the large, Zn induced bend within TF IIIA binding domain of the 5s RNA gene from Xenopus seen previously have been more closely identified. Phased junctions between natural B-form DNA and oligopurine tracts with an altered, Zn dependent conformation seem the most probable cause of the observed bend. There is observed correlation between binding strength of the gene regulatory protein TF IIIA to sequence variants of the 5s RNA gene and the Zn induced bending magnitude of these DNA fragments. The observed bending of the DNA by TF IIIA binding is very probably accomplished by stabilizing the same DNA conformation as Zn binding.

A link between the increased flexibility of the LMM-HMM hinge in assembled minifilaments with carboxy terminal phosphorylation of Acanthamoeba myosin II and actin activated ATPase activity has been uncovered. The same, very distinctive dependence of ATPase activity of copolymeric minifilaments on phosphorylated myosin fraction is also observed in the flexibility of the HMM arms. A fairly stiff spring constant for distortion of the HMM arm attached to the actin appears necessary for myosin function.

The osmotic sensitivity of the B to Z form transition of poly(dG-mdC) has been measured. About 2 water molecules per base pair are released. The interplay of salt concentration and osmotic stress in inducing the transition shows that molecular hydration, not electrostatics, plays the dominant role in determining DNA structure energetics.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-810

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18015-06 LBM

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Histamine Release and Hydration of Granule Matrices

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M.J. Curran	Special Volunteer	LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

This project was transferred to NICHD as of September 30, 1990.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18016-03 LBM

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cell-Cell Fusion Due to Influenza Hemagglutinin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Zimmerberg	Senior Research Investigator	LBM, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was transferred to NICHD as of September 30, 1990.

The Laboratory of Chemistry has been abolished. Projects have been distributed to other areas in NIDDK.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-DK 19001-19 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Reactions and Immunochemistry of Carbohydrates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL.	OTHER

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transferred to LMC 59701 -19

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 19603-15 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analogues of Thyrotropin-releasing Hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Virender M. Labroo	Special Expert	LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19604-21 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Stereopopulation Control in Drug Delivery and Enzyme Simulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Louis A. Cohen	Chief, Sec. Biochem. Mech.	LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19605-15 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Chemistry of Bioimidazoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Sec. Biochem. Mech.	LC, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19606-15 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Halogenated Biogenic Amines in Biochemistry and Pharmacology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Kenneth L. Kirk Chief, Drug Receptor Interactions LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19607-09 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Significance of Ligand Tautomerism in Biorecognition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Louis A. Cohen Chief, Section on Biochem. Mech. LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19608-08 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Functionalized Congeners of Bioactive Compounds

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. Jacobson	Research Chemist	LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19610-04 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prosthetic Groups for Radiolabeling of Functionalized Drugs and Peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: K. Jacobson Research Chemist LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19611-04 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Drugs Acting at Adenosines Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. Jacobson	Research Chemist	LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19612-02 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Aldose Reductase Inhibitors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Louis A. Cohen Chief, Section on Biochem. Mech. LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Bichemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 19613-02 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Virender M. Labroo	Special Expert	LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 19614-02 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Fluorinated Analogues of Bioactive Peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Virender M. Labroo	Special Expert	LC, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Terminated and continued as project in Laboratory of Bioorganic Chemistry.

ANNUAL REPORT OF THE LABORATORY OF CELL BIOLOGY AND GENETICS
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Cell Biology and Genetics carries on a broad program of investigation into exocrine and endocrine secretion and the molecular events regulating these processes. Four specific tissues have been studied: chromaffin cells, which secrete adrenaline, ATP and endogenous opiates; pancreatic beta cells, which secrete insulin; nerve terminals which secrete transmitters and colon carinoma cells which secrete mucins and chloride. These studies were chosen for their promise in elucidating basic defects in stress related diseases such as hypertension and diabetes, and in the genetically based digestive disorder, Cystic Fibrosis. A sustained series of studies were also carried out on the metabolism of ascorbic acid, with an aim to gathering insights into basic questions of nutritional needs and ascorbate functions. The following studies have been performed in pursuit of these goals.

I. Molecular Biology and Biophysics of the Secretory Process in Chromaffin Cells

A. Voltage-Sensitive Calcium Flux into Bovine Chromaffin Cells Occurs Through Dihydropyridine-Sensitive and Dihydropyridine- And ω-conotoxin-Insensitive Pathways

The fluorescent Ca^{2+} indicator FURA-2 was used to characterize the depolarization-related intracellular Ca^{2+} signalling process in bovine adrenal chromaffin cells. Depolarization with high K (10-65mM) gave rise to a very rapid increase in intracellular free Ca^{2+} concentration, which subsequently decayed slowly towards a "plateau". The size of this initial increase varied sigmoidally with the calculated membrane potential, the relationship being described well by a Boltzmann distribution function for a transition between two states (transition potential, -23mV). A dihydropyridine calcium channel agonist {(+)202-791, 1μM} raised intracellular Ca^{2+} concentration further in the presence of 30mM K^+, and it enhanced the initial intracellular Ca^{2+} response to depolarization. Voltage-sensitive calcium channels in chromaffin cells are believed to include the L-type. Several dihydropyridine calcium channel antagonists {(-)202-791, nifedipine, nitrendipine; 1-5μM}, known to be active on L-type channels, caused only modest inhibition of K^+induced increase in intracellular free Ca^{2+} concentration. In addition, ω-conotoxin GVA (1-10μM), a blocker of neuronal N- and L-type calcium channels, reduced the initial increase in intracellular free Ca^{2+} concentration only slightly at 55 mM K^+. Further, the dihydropyridine-insensitive component of the intracellular Ca^{2+} signal was also insensitive to ω-conotoxin, which was otherwise quite active in a central nervous system of the rat *in vivo* preparation. Gd^{3+} (40μM), a potent calcium antagonist in the chromaffin cell, blocked the intracellular Ca^{2+} response to depolarization. When added at different times after K^+ stimulation, however, Gd^{3+} reduced intracellular free Ca^{2+}-concentration to control levels along a slow time course of several minutes. Similar results were obtained when EGTA was added to reduce extracellular Ca^{2+}

concentration to sub-nanomolar levels, in the presence of high K^+. We conclude that bovine chromaffin cells are equipped with at least two classes of voltage-dependent calcium channels, only one of which is likely to be the L-type channel. We also propose that depolarization, in addition to stimulating Ca^{2+} influx, may also lead to enhancement of Ca^{2+} release from an intracellular store.

B. PC-12 Cells Differentiate Into Chromaffin Cell-like Phenotype In Coculture With Adrenal Medullary Endothelial Cells.

Previously we described specific in vitro interactions between PC12 cells, a cloned, catecholamine secreting pheochromocytoma cell line derived from the rat adrenal medulla, and bovine adrenal medullary endothelial cells. We now demonstrate that these interactions induce the PC12 cells to acquire physical and biochemical characteristics reminiscent of chromaffin cells. Under coculture conditions involving direct cell-cell contact, the endothelial cells and the PC12 cells clustered into nests of cells similar to the organization of chromaffin cells seen in vivo. Within 3 days in coculture with endothelial cells, but not with unrelated control cells, PC12 cells synthesized increased levels of [Met]enkephalin. In addition, growing on confluent endothelial monolayers, failed to extend neurites in response to nerve growth factor. Neither medium conditioned by endothelial cells nor fixed endothelial cells could by themselves induce all of these different phenomena in the PC12 cells. These results suggest that under coculture conditions PC12 cells change their state of differentiation toward a chromaffin cell-like phenotype. The rapid, transient increase in the expression of the protooncogene c-fos suggests that the mechanism(s) inducing the change in the state of differentiation in PC12 cells-e.g., by glucoorticoids. We propose that similar interactions between endothelial cells and chromaffin cell precursors may occur during embryonic development and that these interactions might be instrumental for the organ-specific differentiation of the adrenal medulla in vivo.

C. Osmotic Strength differentiates Between Two Types of Calcium Transport Pathways Regulating Catecholamines Secretion From Cultured Bovine Chromaffin Cells

Calcium transport and catecholamine secretion was measured in cultured bovine chromaffin cells. Calcium ions which entered the cells following stimulation with either nicotine or 50 mM KCl (high potassium) triggered catecholamine release, but then inactivated the secretory process. The nicotine and the high potassium-induced calcium transport mechanisms were mechanistically distinct, but functionally dependent on each other. The specific evidence is that whereas the high potassium-induced Ca^{2+} influx was found to be inhibited by hyperosmotic medium, the nicotine-stimulated calcium influx was unaffected under these conditions. High potassium and nicotine-stimulated catecholamine release were also differently affected by hyperosmotic medium. While potassium-stimulated catecholamine release was profoundly inhibited by hyperosmolarity, nicotine-stimulated release was only moderately inhibited. Sequential treatments of cells with nicotine and high potassium, under isotonic physiological conditions, indicate that there is a functional, biochemical communication between the otherwise mechanistically distinct calcium channels. Calcium ions which were found to inactivate these channels may be the basis for such communication.

D. Modulation of Intracellular pH By Secretagogues And The Na^+/H^+ Antiporter In Cultured Bovine Chromaffin Cells

The possible physiological role of cytosolic pH changes in adrenal medullary chromaffin cell secretion was examined by investigating the effects of catecholamine secretagogues on cytosolic pH, which was monitored using the intracellular fluorescent indicator 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Anti-fluorescein antibodies were used to reduce background fluorescence from extracellular 2',7'-bis(2-caroxyethyl)5(6)-carboxyfluorescein (BCECF). Stimulation with both cholinergic agonists (acetylcholine, nicotine) and a depolarizing agent (high K^+) transiently acidified the cytosol of the chromaffin cell. This acidification was antagonized by reducing extracellular Ca^2+ concentration and by Ca^{2+} antagonists (Ca^{2+}, verapamil), indicating that it occurred secondarily to Ca^2+ influx, possibly as a result of exchange of Ca^{2+} ions for protocols across organelle membranes. Taken together with previously published data showing no effect of cytosolic acidification on nicotine-induced catecholamine secretion, these results indicate that secretagogue induced cytosolic pH changes do not represent a casual step in stimulus-secretion coupling of the chromaffin cell. The cytosolic pH recovery to pre-stimulatory cytosolic pH levels was delayed by amiloride and by 5-(N,N-dimethyl)amiloride, at concentrations that otherwise substantially inhibited by cytosolic pH recovery from the rebound acidification induced by a 40-fold sudden dilution of NH_4Cl. This latter type of recovery results from activation of an NA^+/H^+ exchange mechanism. Therefore, the data suggest that Na^+/H^+ exchange is actively involved in the dissipation of the small acid loads generated by secretagogues.

E. Genomic Organization and Chromosomal Localization of the Human Nucleolin Gene

Nucleolin, a eukaryotic nucleolar phosphoprotein, is involved in the synthesis and maturation of ribosomes. To characterize the genomic organization and regulatory sequences of this gene, two overlapping clones containing the human nucleolin gene plus flanking regions were isolated from a genomic library using human nucleolin cDNA. Southern blots of genomic DNA from human, several mammals, chicken, and yeast revealed that the nucleolin gene is well conserved across these species. The gene consists of 14 exons with 13 intervening sequences and spans approximately 11 kilobases of DNA. Analysis of the splice junctions indicated that the amino-terminal domain and the four RNA binding domains plus the nuclear localization signal are split into adjacent exons. Sequences from the 5'-flanking and the first intron contain a high content of GC residues which is consistent with nuceolin being a "housekeeping" gene. Promoter elements include an atypical TATA box (GTTA), one CCAAT box much further from the initiation site, three reverse compliments of CCAAT (ATTGG), and two pyrimidine-rich nucleotide stretches. In addition, this region and the first intron contain numerous potential Sp1, GCF, CRE-fos, GCN, AP-1, AP-2, UCE, and sequences similar to the glucocorticoid receptor binding site. The transcription start site was determined by primer extension and S1 nuclease mapping of RNA from human liver. One Kpn and three Alu repeats were found within two of the middle introns. The 3'-untranslated portion of the gene contain five homology blocks in a 100-base pair region that are highly conserved among human, mouse and hamster genomes. Finally, we have determined that the human nucleolin gene is located on chromosome 2q12 and is present at one copy per haploid genome. A restriction fragment length polymorphism with EcoRI has been detected in the gene.

F. Human Synexin (annexin VII) Polymorphisms: Tissue Specificity and Expression in Escherichia coli

The synexin molecule possess various activities in vitro, which would appear to have great potential relevance to our understanding of important cellular processes, ranging from membrane fusion to ion channel conduction. Construction of the synexin expression plasmid (pTrcFLS) resulted from the ligation of three DNA fragments (pTrc99A) digested with Nco I and Xba I; synexin cDNA digested with Xma I engineered at position 69 and Spe I; and a double stranded oligomer with CATGTCATAC and CCGGGTATGA have Nco I and Xma I which have compatible ends) (pTrc99A and E. coli RB 971 were gifts from Dr. E. Amann. Similar procedures were followed to make the pTrcR4S and the pTrcEIIS plasmids, which encoded proteins containing only the four C-terminal repeats of synexin and a chimaera with the N-terminus of endonexin II plus the C-terminus of synexin respectively. In the latter two cases, pTrc99A Nco I/Xba I, and synexin Xmn I/Spe I fragments were ligated to double stranded oligomers using sequence information from the respective cDNAs [9,12]. Extracts of all three recombinant plasmids expressed in E. coli RB 971 contained proteins of the appropriate molecular weight and were immunoreactive with polyvalent anti-synexin antisera by western analysis. Recombinant human synexin purified from E.

coli was tested for its ability to aggregate chromaffin granules. Recombinant synexin indeed caused granules to aggregate, and furthermore, to do so in a manner dependent on the concentrations of both calcium and added protein. The calcium dependent for both natural and recombinant synexin proved similar. Finally, we also found that recombinant human synexin could form calcium channels in phosphatidylserine bilayers formed at the tip of a patch pipette. These data together verify that the cDNA cloned on the basis of specific amino acid sequences is, in fact synexin, and that the recombinant molecule from E. coli expresses the properties of the naturally occurring molecule from human tissue.

G. Pertussis Toxin Stimulation of Catecholamine Release from Adrenal Medullary Chromaffin Cells: Mechanism May Be by Direct Activation of L-Type and G-Type Calcium Channels

We have previously shown that pertussis toxin (PTX) stimulates delayed onset. $[Ca^{2+}]_i$,-dependent catecholamines (CA) release from bovine chromaffin cells. We now show that this effect of PTX is inhibited in part (50%) by dihydropyridine Ca^{2+}-channel antagonists niludipine and nifedipine, and is potentiated by the dihydropyridine Ca^{2+} channel agonist Bay K-8644. We and others have shown that pretreatment of chromaffin cells with PTX results in enhanced catecholamine secretion in response to high $[K^+]_o$, nicotine and muscarine, and here we extend these observations by showing that toxin pretreatment also enhances the secretory response to $[Ba^{2+}]_o$. All these data are consistent with the concept that PTX may act on Ca^{2+} channels. To examine the possibility of a direct action of the toxin on the voltage-gated L-type Ca^{2+} channel known to be present in these cells, we studied the effects of the toxin on whole cell Ca^{2+} currents. We found and report here that spontaneous electrical activity was considerably increased in PTX-treated cells. Our measurements of whole cell inward Ca^{2+} currents indicate that the underlying mechanism is a marked shift of the activation curve of the L-type Ca^{2+} current along the voltage axis towards more negative potentials. While treatment of the cells with PTX had no effect on L-type Ca^{2+}-channel conductance (6 nS/cell at 2.6 mM $[Ca^{2+})$, PTX evoked the activation of a new class of Ca^{2+}-selective channels (5 pS in 25mM $[Ca^{2+}]_{pipet}$), which are rather insensitive to membrane potential. We have termed these G-type calcium channels. These data suggest that treatment with PTX not only increases the probability of L-type Ca^{2+}-channel activation at more negative potentials, but also increases the probability of opening of an entirely new, voltage-independent, Ca^{2+}-channel. These actions of PTX should promote Ca^{2+} entry and might explain the stimulation by the toxin of CA secretion from medullary chromaffin cells in culture.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 21008-25 LCBG

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)
Cytogenetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: J.H. Tjio

COOPERATING UNITS (if any) UMD-New Jersey Med. School, Newark, NJ (E. Scott Raveche); Univ. California, Berkeley (G. Brecher); Ernst Moritz Arendt Universitat, Greifswald, Germany (F. Herrmann); Humboldt Universitat, Berlin, Germany (S. Santibanez).

LAB/BRANCH
Laboratory of Cell Biology and Genetics

SECTION
Cytogenetics

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER.
2	1	1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. Sex reversal studies and the effect of various steroid hormones in fish. No clearcut sex reversal has been observed after steroid treatment.

2. Analysis of clonal hyperdiploid CD+B cells in mice. The finding that IL-10 is increased in clones of CD5+B cells which in many respects are malignant, offers the possibility that anti-IL-10 may prevent the development of these clones and therefore be useful in the treatment of CLL. The hyperdiploid CD5+B cells are not only a model for such autoimmune diseases as rheumatoid arthritis and Sjogren's syndrome, they are very similar to the malignant cell in virtually all chronic lymphocytic leukemias. In all of these states, immunosuppression of normal B cell function is observed. The immunosuppression observed may be due to the IL-10 which is produced.

3. Post and prenatal diagnosis of chromosomal aberrations in man.

4. Cytotaxonomic studies of the genus Macropodus and Trichogaster.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 21019-09 LCBG

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of hormone and transmitter secretion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our work continues to focus on the processes leading to fusion between granule and plasma membranes during exocytosis in cells, including chromaffin cells, beta cells from islet of Langerhans, nerve terminals and mucin secreting human colon carcinoma cells. The contact and fusion event during exocytosis may be controlled by the protein synexin. We have recently cloned and sequenced the cDNA for this protein, and learned that it is a member of the annexin gene family. We have expressed synexin in E. coli and have found the recombinant protein to express both fusion and channel properties. Secretion mechanisms have also been studied in intact chromaffin cells, in which evidence has been found to support the concept that calcium and barium promote secretion by separate mechanisms. The acetylcholine receptor in chromaffin cells has been found to be different from the classical nicotinic receptor, inasmuch as it has unexpected muscarinic character. A novel calcium channel has been discovered in human beta cells, which responds to glucose and related metabolites. This channel is named by us the "G-type calcium channel, and may play a central role in glucose activation of islets. Glucose induced insulin release is modulated by acetylcholine, and pharmacological analysis reveals that the muscarinic receptor type is M1 rather than M2. The adrenal medulla has provided a tractable system to study the interaction between endocrine cells and adherent capillaries. Endothelial cells have MAO-A, exclusively, and various steroids affect expression of this enzyme, without affecting the MAO-B which is exclusively found in chromaffin cells. A new method for measuring ascorbic acid using HPLC and coulometric electrochemical detection has been developed. The method allows measurement of the vitamin, not only in cells such as leucocytes and chromaffin cells, but also human plasma. Two transporters for ascorbic acid have been detected in human neutrophils. Millimolar concentrations of ascorbic acid have been detected in unbound form in both human neutrophils and monocytes.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. POLYAMINES

The aim of our work has been to elucidate the physiological function of the polyamines, and their role in growth and differentiation. For this purpose we have constructed a variety of mutants in both *Escherichia coli* and *Saccharomyces cerevisiae*, and have studied the phenotypic effects of the depletion of the intracellular polyamines. In addition, we have studied the enzymes involved in the biosynthesis of these amines, and their regulation *in vitro* and *in vivo*.

We have now extended these studies by observing the phenotypic effects of polyamine depletion in null mutants. We have prepared such null mutants in both *E. coli* and in *S. cerevisae* by deletion and disruption of the chromosomal genes for the biosynthetic pathways. The advantage of these newly-constructed mutants is the certainty that no traces of enzyme or polyamines are present.

Null mutants have been constructed in the genes for both ornithine decarboxylase (*spe1*) and *S*-adenosylmethionine decarboxylase (*spe2*); both of these enzymes are required for the biosynthesis of spermidine and spermine. We have used these null mutants to show that *S. cerevisiae* has an absolute requirement for polyamines for growth and that spermidine and/or spermine is absolutely necessary for growth even in the presence of excess endogenous or exogenous putrescine.

. . . . . Drs. D. Balasundaram, Q.-W. Xie, C. W. Tabor, and H. Tabor

Growth of the Δ*spe2* null mutant (lacking spermidine and spermine) showed several surprising phenotypes that had not been observed with the point mutants we had previously prepared:

1. There is complete cessation of growth in the absence of amines. This is true even though these cells have a very high level of putrescine.
2. Surprisingly this absolute requirement for spermidine and/or spermine is true only for aerobic growth. There is no requirement for these amines if the growth is under anaerobic conditions.
3. The cells show a marked increase in size (20X the normal volume).
4. The cells contain many large vesicles throughout the cytoplasm.
5. Budding is markedly reduced.
6. Chitin rings are not formed and chitin is distributed abnormally in the cell.
7. Actin is present but fails to accumulate in the normal locations.
8. A most striking and unexpected finding was that marked defects occur in mitochondrial structure and function after prolonged growth in the absence of amines. Even after the cells are transferred to rich media or media containing polyamines, the cells cannot grow on glycerol. Even though these cells cannot grow on glycerol, they can still grow well on glucose, but the colonies are "petite".

These observations represent the first time that an absolute requirement for spermidine and/or spermine has been demonstrated in yeast. The morphologic effects are striking and resemble those seen in certain cell cycle (cdc) mutants, suggesting a possible role for these amines in critical steps of the cell cycle. The findings with mitochondria indicate an important role for polyamines in the biogenesis and/or maintenance of mitochondria.

. . . . . Drs. D. Balasundaram, C. W. Tabor, and H. Tabor

The nucleotide sequence of several *spe1* point mutants was determined, and the specific sites of the mutations defined. Using a strain with a mutation in the amino-terminal region of the gene and another strain with a mutation in the carboxyl terminal region, with both *in vivo* and *in vitro* protein synthesis systems we demonstrated complementation, i.e., the formation of active ornithine decarboxylase.

. . . . .Drs. Q.-W. Xie, H. Tabor, and C. W. Tabor

Our previous studies on the *speED* operon of *E. coli* showed that there were three open reading frames: (1) an open reading frame of unknown function in the upstream portion of the operon; (2) the gene *speE*, coding for spermidine synthase; and (3) the gene *speD*, coding for S-adenosylmethionine decarboxylase. Null mutations were constructed in each of these three regions but were found to have little, or no effect, on the growth rate of these strains, even after these strains were grown in amine-free media. A sensitive HPLC method was developed for the detection of small amounts of amines and was used to confirm that no spermidine was present in these cultures.

Thus, *E. coli*, in contrast to the findings summarized above for *S. cerevisiae*, can grow indefinitely in the absence of spermidine. The only defect that we have observed in spermidine-deficient mutants of *E. coli* is a marked increase in the toxicity of paraquat, a compound known to produce superoxide in air (cf. Annual Report 1990).

. . . . . Drs. Q.-W. Xie, H. Tabor, and C. W. Tabor

II. YEAST RNA VIROLOGY

-1 *Ribosomal Frameshifting*. We developed vectors that can be used for a simple plate assay to measure the efficiency of -1 ribosomal frameshifting. This system is now being used to screen for drugs affecting this process. Ribosomal frameshifting is used by most retroviruses to make *gag-pol* fusion proteins, but is not known to be needed by eukaryotic cellular genes. We also showed that either increased or decreased rates of frameshifting adversely affect M satellite virus replication.

. . . . . Drs. J. Dinman and R. B. Wickner

Activities of the pol domain. The *pol* ORF of L-A has the consensus sequence patterns typical of the RNA dependent RNA polymerases of (+) strand RNA viruses. We have precisely defined the area around these sequences necessary for viral replication using localized mutagenesis. We have also partially localized the region necessary for the single-stranded RNA binding activity. The replication activity requires two sites on the RNA: an internal site where the polymerase binds first and a 3' end site where polymerization begins. We now show that the polymerase uses a "looping" mechanism to grab the 3'end site after binding at the internal site.

. . . . .Drs. J. C. Ribas, T. Fujimura and R. B. Wickner

MAK3 is an N-Acetyl transferase blocking the N-terminus of the L-A major coat protein. mak3-1 mutants cannot replicate L-A or M virus. We cloned the MAK3 gene using chromosomal walking based on the genetic map position of the gene. The sequence showed that MAK3 is highly homologous to various N-acetyltransferases, particularly the *riml* gene of *E. Coli* encoding an enzyme that acetylates the N-terminus of a ribosomal protein. While we showed that MAK3 does not acetylate a ribosomal protein in yeast, we found that the major coat of L-A and M virus particles is blocked (acetylated) in MAK^+ strains, but unblocked in mak3-1 strains. The unblocked major coat protein is apparently rapidly degraded and does not assemble into viral particles. The MAK3 gene is not essential for cell growth, but is necessary for rapid growth on non-fermentable carbon sources.

. . . . .Drs. J. C. Tercero and R. B. Wickner

MAK10 expression is glucose-repressed with control sequences inside another gene. mak10 mutants cannot replicate L-A or M virus. We cloned the MAK10 gene by chromosome walking based on its genetic map position. MAK10-LacZ fusion genes were used to measure its expression. MAK10 expression is low in glucose media and high in glycerol or ethanol media. Cells lacking mitochondrial DNA, and thus able to grow only on glucose, have undetectable levels of MAK10 expression. Mutants in tup1 or cyc8, two genes necessary for glucose repression, have very high levels of MAK10 expression. Others have previously shown that L-A virus replication is glucose-repressed. We hypothesize that the glucose repression of MAK10, a gene whose product is necessary for L-A replication, is responsible for this effect. Preliminary localization of the controlsequences of MAK10 show that they are, surprisingly, inside an adjacent long open reading frame.

. . . . . Drs. Y.-J. Lee and R. B. Wickner

SK12 has homology to helicases; SK18 has repeat sequences typical of β-transducin. We have cloned and sequenced the SKi2 gene and find that it encodes a 140 kDa protein with the sequence patterns characteristic of RNA helicases. Our sequence of the SK18 gene shows that it has two copies of a repeat pattern first identified in β-transducin. Both SKi2 and SK18 (as well as SK13 which we previously characterized) are essential to the cell only for repression of viral replication.

. . . . . Drs. W. R. Widner and R. B. Wickner

20S RNA Sequence. We recently showed that 20S RNA, a species whose synthesis is induced by transferring cells to acetate medium, is a circular ssRNA autonomous replicon. Earlier reports claimed that each 20s RNA was associated with 20 copies of a 23 kDa protein. We have now shown that this 23 kDa protein is the Hsp26 heat-shock protein and that there is no association, either structural or functional. We have cloned and sequenced most of 20S RNA and find that it encodes a 90 kDa protein with some homology to RNA-dependent RNA polymerases and potential RNA-binding sites. We have detected the 90 kDa protein *in vivo* in cells whose synthesis of 20S RNA was induced.

. . . . . Drs. Y. Matsumoto, W. R. Widner and R. B. Wickner

III. NUCLEIC ACIDS

L1 DNA (long interspersed repeated DNA, LINE 1 DNA) is a ubiquitous feature of mammals and comprises at least 10-20% of their genomes. L1 elements contain a promoter-like sequence at the left end, two highly conserved open reading frames (ORFs), and a guanine-rich polypurine/polypyrimidine sequence near the right terminus. Amplification of L1 elements has occurred repeatedly during mammalian evolution and invasion by L1 elements into new sites is a frequent cause of polymorphism in mammals including humans. This can occur during the lifetime of the individual as was dramatically illustrated by a case of factor VIII deficiency due to L1 insertion in the factor VIII gene of a child whose parents both had the normal gene. We have been studying the L1 family of rats and describe below our recent findings.

We previously showed that the polypurine:polypyrimidine sequence at the right end of the rat L1 element adopts a series of abnormal DNA structures *in vitro* that may explain some of the properties of this sequence. These include: (1) its ability to induce single strand uptake by contiguous duplex sequences *in vitro* in a reaction that mimics early steps in the recombination process; and (2) its sensitivity to nucleases isolated from mammalian cells. We have suggested that these properties *in vitro* may explain the propensity of polypurine:polypyrimidine stretches to act as a hotspot for recombination *in vivo*

We also showed that the L1 non-B structures competes with other types of non-B DNA structures for limiting amounts of supercoil energy. Therefore, the L1 polypurine sequence may affect a variety of other processes *in vivo* that are sensitive to the level of supercoil energy (*e.g.* gene expression or DNA replication).

We are now examining the possible effects of the L1 polypurine:polypyrimidine region *in vivo*. We have shown that this region decreases the replication of plasmids bearing the SV40 replicon in monkey COS cells by over 90% and that it decreases the amount of chloramphenicol acyl transferase (CAT) produced from CAT gene fusions with certain eukaryotic promoters. In parallel studies we have shown that homoguanine stretches enhance the mutation rate of adjacent DNA sequences *in vivo*.

. . . . . Drs. K. Usdin and A. V. Furano

We previously showed that the left-most 610 bp of rat L1 DNA is enriched in the dinucleotide, CpG, which is characteristic of the regulatory or promoter-like region of certain genes, and that it can stimulate the activity of a reporter gene fused to it. We now have found that the L1 regulatory region enhances the activity of regulatory sequences that are located 5' to it. In addition to activating cryptic promoters it also strongly stimulates both the early and late SV40 promoters. The latter experiments were carried out using COS cells that were transfected with plasmids bearing the SV40 origin/promoter sequence in either the "early" or "late" promoter orientation (relative to the reporter gene). So far, we have studied the "late" promoter orientation plasmid in most detail and found that the L1 sequence has two effects: It increases the replication of this plasmid, which otherwise replicates quite poorly compared to the "early" orientation plasmid, to normal levels, and it causes a massive stimulation of the "late" promoter without altering the transcriptional start site. Interestingly, most of these transcripts do not extend into the reporter gene but terminate within the L1 regulatory region itself. We are now determining whether termination is due to a block in transcription or to an RNA processing event, and whether such terminated transcripts are also produced from the L1 stimulated "early" promoter.

. . . . . Drs. B. Hayward, E. Pascale, and A. V. Furano

The L1 promoter is unusual in that it does not contain binding sites for any of the general transcription factors. Nonetheless, DNA binding experiments show that three different regions of the L1 regulatory sequence can form specific complexes with partially purified nuclear factors present in the COS cell line used for the above experiments. Since deletion of the binding site for the first region increases both the basal activity of the regulatory region as well as its stimulation of the SV40 promoter, we conclude that somatic cells contain protein factors that repress the activity of the L1 regulatory sequence. Interestingly, this region contains a perfect version of a recently described factor binding site found in the regulatory region of the rat chymotrypsin gene. Deletion of region three greatly diminishes the activity of the L1 regulatory region and we assume that this region binds a transcriptional stimulatory factor.

. . . . . Drs. B. Hayward and A. V. Furano

To date neither of the putative proteins encoded by the two L1 open reading frames has been identified. To do this we have developed a series of cloning vectors to facilitate the isolation of functional L1 ORFs from genomic DNA. We have also fused the ORF I sequence to the maltose binding protein and are in the process of optimizing conditions for the production of large amounts of this fusion protein from which large amounts of pure ORF I protein can be prepared by affinity chromatography on a maltose column followed by specific proteolytic cleavage of the purified product.

Screening for functional ORF encoded proteins is complicated by the fact that most of the L1 elements in cells are defective. Computer analysis of the putative ORF II protein shows that it has motifs typical of reverse transcriptases. Therefore, we are devising a method to screen for reverse transcriptase in *E.coli*. The method is potentially very sensitive since only bacteria containing an active reverse transcriptase will survive the selection procedure. We are currently testing this method which could be also used to screen for any reverse transcriptase.

. . . . . Drs. B. Hayward, K. Usdin, and A. V. Furano

The fact that the L1 families in different mammalian species are homologous for most of their length but contain completely distinct regulatory regions indicates that each present day L1 family is the product of an independent amplification event. Although L1 DNA was present in mammals before their radiation 80 million years ago, modern genomes were thought to lack highly amplified ancestral L1 elements. This suggested that extensive amplification of L1 families is a recent event in mammalian evolution, or that ancestral L1 elements were cleared from the genome as new copies were generated.

However, we recently showed that the amplification of the present day rat and mouse L1 families was preceded by an earlier amplification of an ancestral L1 family. This occurred about 11 million years ago in the ancestor of modern murine rodents, and the relics of this amplification have persisted in modern murine genomes. We have since shown by DNA sequence analysis that both the modern mouse and rat L1 families were derived from the ancestral murine L1 family and that L1 amplification appears to be episodic rather than continual.

We further showed that analysis of ancestral amplification events could provide a novel method for examining the evolutionary relationships between modern species. To this end we began a collaboration with Dr. Francois Catzeflis (Institute of Evolutionary Science in Montpellier, France), a molecular phylogenist working in rodent taxonomy. Our collaborative studies have already resolved several problems in rodent phylogeny, demonstrating the utility of this technique, and in addition, have revealed several novel aspects of L1 evolution.

The finding that the products of an ancestral L1 amplification persist in the genome suggested that L1 families might account for far more of the genome than the 5-10% previously estimated from the copy number of modern L1 families. We therefore prepared oligonucleotides from several highly conserved regions of ORF II and found by hybridization to genomic DNA that L1 DNA alone accounts for at least 40% of the rat genome. This finding indicates that L1 has had a major impact on shaping the mammalian genome and has important implications for, among other things, the human genome project.

. . . . . Drs. A. V. Furano, E. Pascale, B. Hayward, K. Usdin, and F. Catzeflis

We have found that a large number of routine record keeping tasks and analysis that involve nucleotide or protein sequences, recombinant DNA clones, laboratory methods and reagents, etc. can be most accurately and efficiently handled by a computer. This approach is especially useful if various members of the same laboratory are able to share the creation and use of the databases containing the above items on a computer network. Since no commercially available software was suitable for this purpose we devised a set of data management tools for molecular biologists that runs on a Macintosh™ network.

. . . . . Dr. K. Usdin

We are studying the *E. coli* bacteriophage T4 as a model system for duplex DNA replication. Efficient DNA replication *in vitro* requires at least ten purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), gene 32 helix destabilizing protein, the gene 44/62 and 45 polymerase accessory proteins, the genes 41 and 61 proteins that function together as a primase and as a DNA unwinding enzyme (helicase), gene 59 protein that stimulates the primase-helicase, RNase H, and DNA ligase.

Assembly of the polymerase and accessory proteins on the primer-template. Protein-DNA cross-linking has been used to determine how and where T4 DNA polymerase and the 44/62 and 45 polymerase accessory proteins assemble on the primer strand of a primer-template. Using a system in which a photoactivatable aryl azide can be moved stepwise through a thirty base region of a primer annealed to a long single-stranded circular template, we have found that the accessory proteins and 32 protein can be cross-linked to specific positions of the primer in the presence of the non-hydrolyzable analogue ATPγS. These studies showed that 32 protein bound to the primer-template, with or without ATP, but that either ATP or a non-hydrolyzable analogue was required to bind the three accessory proteins. ATP hydrolysis changed the position and intensity of cross-linking to each of the accessory proteins, and was essential to assemble a complex that included polymerase as well as the other proteins. These studies suggest that the initial binding of the three accessory proteins to a 32 protein-covered primer-template is followed by ATP hydrolysis, binding of polymerase, and movement of the accessory proteins to yield a complex capable of processive DNA synthesis.

We are currently using both gel mobility shift assays and cross-linking to determine how large a template strand is required for assembly of these proteins. The gel mobility shifts indicate that 32 protein binds to a 36/60-mer primer-template without additional proteins or cofactors. Binding of the accessory proteins can be demonstrated by this technique if all three of these proteins, 32 protein and ATPγS are present. Polymerase does not interfere with the formation of this complex and there is no indication that polymerase can add to it. At least two different gel-shifted bands are found when polymerase alone is added to the 36/60-mer, and additional bands are evident with both polymerase and the 32 protein, with or without ATP. None of the bands are changed by adding the accessory proteins. These experiments suggest that either the accessory proteins are moving too rapidly in the presence of ATP to form a stable complex with the DNA, or that the 24 base single-stranded region of the template is too short for the proper assembly of a complex including both polymerase and the accessory proteins. The latter interpretation is favored by our findings that the 45 protein cannot be cross-linked to a 34-mer annealed to a complementary 60-mer under conditions where it is cross-linked to the same 34-mer annealed to a long circular template, and that the accessory proteins do not prevent the pause bands indicative of copying by polymerase alone on the 34/60-mer primer-template. We are constructing larger templates to determine the optimum length for assembly of the complete complex of polymerase and the accessory proteins, and then plan to make templates containing cross-linkable residues to determine the location of each protein on the template strand.

. . . . . Drs. T. L. Capson and N. G. Nossal, in collaboration with Dr. S. J. Benkovic (Pennsylvania State University)

Cloning, purification, and characterization of the T4 gene 59 protein. T4 mutants in gene 59 are conditionally lethal and are particularly defective in DNA repair. The gene is located between those for RNase H and the 32 protein. We have cloned gene 59 under the control of the T7 promoter, and have devised an efficient purification procedure yielding homogeneous protein in two steps. We find that gene 59 protein stimulates both the primase and helicase activities of the gene 41 and 61 primase-helicase, in agreement with the preliminary report of Hacker, Barry, and Alberts (J. Cell. Biochem. **13D** 87 (1989)). Kinetic studies with forked DNA molecules suggest that the rate of unwinding of individual molecules by 41 protein is not increased, but that more molecules are unwound in the early stage of the reaction.

. . . . . Dr. N. G. Nossal

Protein-protein interactions required for strand displacement synthesis. Mutations in T4 DNA polymerase can decrease its ability to interact with the proteins that allow strand displacement synthesis with the wild type polymerase. The independently isolated tsCB120 and tsL141 polymerase mutants were originally characterized as temperature sensitive for DNA replication and antimutators at the permissive temperature. Linda Reha-Krantz and her colleagues have shown that both of these mutants result from a change of alanine to valine at position 737, and can be suppressed by a second mutation of leucine to phenylalanine at 771. We have constructed the tsL141 and L771F single mutants and the double mutant by site-directed mutagenesis of the cloned polymerase gene. The purified tsL141 polymerase is stimulated by the accessory proteins and 32 protein, but is arrested at pause sites more frequently than the wild type on single-stranded DNA templates. The L771F polymerase resembles the wild type, and the double mutant is arrested much less that the tsL141 polymerase. Even at the permissive temperature, the tsL141 polymerase carries out practically no strand displacement (leading strand) synthesis on a forked DNA template with the accessory proteins, 32 protein, and the primase-helicase. We find that addition of the gene 59 protein, which stimulates the primase-helicase, is absolutely required for strand displacement synthesis with the tsL141 polymerase. The positions of the tsL141 and L771F mutations are C-terminal to the regions where T4 polymerase shows amino acid sequence similarities with the eukaryotic DNA polymerase α family of polymerases, and may be in a region important for its interactions with the other T4 DNA replication proteins.

. . . . . Drs. P. Spacciapoli and N. G. Nossal

Transcription from a bacteriophage T4 middle promoter using T4 motA protein and phage-modified RNA polymerase. During infection by phage T4, the use of specific promoter sequences for the transcription of early, middle, and late genes correlates with different phage-induced modifications to the host polymerase. We are studying the expression of a cluster of T4 genes (5' → 3'): *X.1* (DNA endonuclease, [see below]), *uvsX* (recombination protein), 40 (stimulates head formation), and 41 (DNA replication protein, part of the primase-helicase) as a model for understanding how the phage regulates middle RNA.

The T4 motA protein is required *in vivo* for expression from T4 middle promoters. We have developed an *in vitro* system which transcribes from a T4 middle promoter located upstream of the *uvsX* gene. This system is dependent on the T4 motA protein and on host RNA polymerase isolated 4 min after T4 infection (the start of middle gene expression). Host RNA polymerase isolated from an uninfected cell catalyzes the synthesis of minor amounts of RNA from this promoter, but this synthesis is not motA-dependent. Gel shift assays demonstrate that in the absence of motA protein, either host or middle polymerase can form a heparin-resistant complex with this promoter. Addition of motA protein results in a unique, slower-migrating species with either polymerase, but only the complex with middle polymerase is resistant to heparin challenge. Thus, motA protein may be needed to form a polymerase-DNA open complex that is competent for transcription, and the T4-induced changes to the polymerase may be needed to stabilize this complex.

. . . . . Dr. D. M. Hinton

Identification of a family of bacteriophage T4 genes encoding proteins similar to intron Open Reading Frames (ORFs) of fungi and phage. The biological function of the T4 *X.1* gene is unknown since a T4 *X.1* mutant is normal for phage growth and UV repair. We have found that the X.1 protein shares a tremendous degree of similarity with 4 other T4 open reading frames also of unknown function and that together these five ORFs share regions of homology with the endonuclease encoded by the T4 *td* intron and with putative endonucleases of group I introns present in fungi. These endonucleases are required for the movement ('homing') of intron DNA into its intronless gene. We have demonstrated *in vitro* that like the *td* intron-encoded endonuclease, X.1 protein is a Mg^{++}-dependent DNA endonuclease with preferred sites for cutting DNA. We speculate that X.1 and the 4 similar T4 ORFs represent a family of endonucleases related to the endonucleases present in mobile group I introns.

. . . . . Drs. M. Sharma and D. M. Hinton

IV. MEMBRANE STUDIES OF *ESCHERICHIA COLI*

Aldoheptose Biosynthesis. E. coli K-12 aldoheptose mutants, carrying the *cysE-pyrE* linked mutation *rfaD* or *rfa-2*, were previously isolated and genetically characterized. The *rfaD* phenotype includes increased permeability to a large number of hydrophobic antibiotics, in addition to altered LPS synthesis. The *rfaD* gene initially identified in the Clarke-Carbon Genomic Bank was cloned. The precise location of the *rfaD* gene on a 1.3-kilobase SspI-HpaI fragment has been determined. The *rfaD* gene and the flanking regions have been completely sequenced, and the coding and regulatory regions have been defined. The location of the *rfaD* gene on the physical map of the *Escherichia coli* chromosome has been resolved. RfaD plasmids express *in vivo* and *in vitro* a protein with the molecular weight of 37,000. The protein, ADP-L-glycero-D-mannoheptose-6-epimerase, has been purified to homogeneity and partially characterized. N-Terminal analysis of purified ADP-D-glycero-D-mannoheptose-6-epimerase confirms the first 34 amino acid sequence deduced from the nucleotide sequence of the *rfaD* gene coding region. The rfaD protein contains the fingerprint sequence of the ADP-binding βαβ-fold of NAD-binding proteins, near its N-terminus. The *rfaD* gene studies have been extended to include the clinical pathogen *Pseudomonas aeruginosa*. The second *cysE-pyrE* linked mutation, designated *rfa-2*, resulted in heptoseless LPS (chemotype Re) and increased permeability to hydrophobic and hydrophilic agents. We have cloned the *rfa-2* gene, resolved its sequence and flanking nucleotides, as well as determined its physical location on the *E. coli* chromosome. The *rfa-2* gene encodes a 36Kd protein. Interspecific complementation studies demonstrated that the *E. coli* K-12 *rfa-2* gene complements the chemotype Re LPS mutant of *S. typhimurium*, designated *rfaC*.

. . . . . Drs. L. Chen, L. Ding and W. G. Coleman, Jr.

V. ENZYME MECHANISM AND PROTEIN STRUCTURE

Tryptophan synthase is a multienzyme complex that catalyzes the final two reactions in the biosynthesis of L-tryptophan. Our work is aimed at using tryptophan synthase as a model system to understand how protein-protein interaction affects catalysis, metabolite channeling, and ligand-dependent site-site interactions.

Three-Dimensional Structure of the Tryptophan Synthase $\alpha_2\beta_2$ Multienzyme Complex from Salmonella typhimurium We have previously determined the structure of the tryptophan synthase multienzyme complex by x-ray crystallography at 2.5 Å resolution. The structure has now been refinded to higher resolution. The presence of a tunnel 25Å long that connects the active sites is being investigated in greater detail. The structure serves as a framework for choosing residues for further investigation by site-directed mutagenesis. Crystallographic analysis of mutant and wild type forms of the enzyme in the presence and absence of ligands is in progress.

. . . . . Drs. E. W. Miles and S.A. Ahmed with Drs. C. C. Hyde and D. R. Davies (LMB, NIDDK)

Site-Directed Mutagenesis of the Tryptophan Synthase $\alpha_2\beta_2$ Complex. We are using recombinant DNA techniques to modify the trpA and trpB genes from *Salmonella typhimurium*. We are investigating the catalytic roles of several amino acid residues that are located in the active site of the β subunit or line the tunnel. Mutation of these residues modifies the substrate and reaction specificity and leads to substrate-induced inactivation. The results clarify the origin of reaction specificity and the control of reaction specificity by protein-protein interaction. Studies of the effects of mutation and of proteolytic cleavage of a flexible loop in the α subunit demonstrate that this α subunit loop plays a critical role in regulating the activity of the β subunit. We plan to examine the effects of mutation on protein conformation by x-ray crystallographic analyses.

. . . . . Drs. E. W. Miles, S. A. Ahmed, A. M. Kayastha, X. Yang, S. Ruvinov, and Z. Lu

Detection and Identification of Intermediates in Reactions of Tryptophan Synthase. The pyridoxal phosphate coenzyme provides a convenient spectroscopic probe to directly monitor catalytic events at the active site of the β subunit. We are using steady-state and rapid-scanning stopped-flow spectroscopy to detect enzyme-substrate intermediates in reactions of tryptophan synthase and to determine the rates of formation and decay of transient intermediates. We find that the equilibrium distribution of intermediates is pH dependent and is under allosteric regulation. Mutations of residues in the active site of the α subunit affect the reactivity of substrates at the active site of the β subunit 25 Å distant. These results shed light on the mechanism of allosteric regulation and of channeling of indole. A mutation at the active site of the β subunit also alters the reaction and substrate specificity.

. . . . . Drs. E. W. Miles, A. M. Kayastha and Y. Sawa with Drs. P. Brzovic and M. F. Dunn (University of California, Riverside) and A. Mozzarelli and G. L. Rossi (University of Parma, Italy).

Kinetics of Substrate Channeling. Single turnover chemical quench-flow and stopped-flow methods provide the first direct evidence that indole is channeled through the tunnel that connects the active sites of the α and β subunits of tryptophan synthase. Ligands and a mutation at the β site alter the rates of channeling and of intermediate steps. The results demonstrate that the reaction of serine at the β site modulates the formation of indole at the α site. These findings elucidate how intersubunit communication regulates the reactions catalyzed at the active site of each subunit. We are now applying these techniques to investigate the effects of mutations designed to block or restrict the tunnel.

. . . . . Drs. E. W. Miles and X. Yang with Drs. K. S. Anderson (Yale University New Haven, CT) and K. A.Johnson (Pennsylvania State University, University Park, PA).

Thermal Unfolding of Tryptophan Synthase. The effects of subunit interaction and of mutation on the stability and thermal unfolding of tryptophan synthase are investigated by differential scanning calorimetry, by temperature dependent UV/VIS spectroscopy, and by temperature dependent circular dichroism spectroscopy. Calorimetric titration studies determine subunit association constants and the effects of mutation and of ligands on the strength of subunit association.

. . . . . Drs. E. W. Miles and H. Kanzaki with Drs. P. McPhie (LBM, NIDDK), A. Ginsburg (LB, NHLBI), and Dr. K. Yutani (Protein Institute, Osaka University, Japan)

The technique of sedimentation equilibrium has been employed to study the interactions between purified GPIIIb/IIa, the platelet fibrinogen receptor, and fibrinogen in the absence and presence of calcium and solubilizing detergents. The receptor (MW ca. 220K) is largely globular and hydrophilic except for a lipophilic tail that resides within the platelet plasma membrane and anchors the receptor to it. In the absence of solubilizing detergents, the lipophilic tails aggregate in solution to form receptor clusters (so-called "rosettes") having a broad distribution of molecular weights. We have not been able to detect binding of fibrinogen to purified receptor in the presence of detergents under conditions that would have detected associations with an equilibrium association constant exceeding 10^4 M^{-1}. In the absence of detergent, we do detect significant association of fibrinogen and receptor, but the quantitative analysis of binding is complicated by the heterogeneity of the receptor under these conditions.

. . . . . Drs. G. Rivas and A. Minton

A mathematical model has been developed that enables us to simulate the time course of separation of macromolecules on a self-forming density gradient of CsCl in a preparative ultracentrifuge. The model contains information about the hydrodynamic and thermodynamic properties of each of the components, and then permits the user to numerically solve the sedimentation-diffusion equation governing flow of molecular species in a centrifugal field. Algorithms have been devised to permit rapid solution of this equation on personal computers at rates that are more than 100-fold greater than actual elapsed time on the centrifuge. Comparisons with experimental data were carried out in order to refine model approximations, and in recent months time predictions of the model have been found to be in good agreement with a variety of experiments, performed subsequent to the simulations, on the separation of linearized and closed circular plasmid DNA in the presence of ethidium bromide.

. . . . . Drs. A. Minton with A. Furst and L. Holladay (Beckman Instruments, Palo Alto, CA)

Glutathione and an uncharacterized other substance are indicated to be regulators of the activity of a valyl-tRNA synthetase. The protein complex in which the regulation occurs is suggested to be the "tip of an iceberg" representing a vast cytoplasmic apparatus that orchestrates protein biosynthesis and cell development. This system could be the seat of function of oncogene proteins that on mutation cause the regulatory malfunctions of cancer.

. . . . . Dr. S. Black

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and similarity of these sequences. The property of uniqueness (the occurrence of a *small* peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under continued investigation.

. . . . . Drs. H. A. Saroff and E. Mihalyi

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 23,140-33 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry of Sulfur-Containing Compounds

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Simon Black, Ph.D.	Chemist (Research)	LBP NIDDK

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Glutathione and an uncharacterized other substance are indicated to be regulators of the activity of a valyl-tRNA synthetase. The protein complex in which the regulation occurs is suggested to be the "tip of an iceberg" representing a vast cytoplasmic apparatus that orchestrates protein biosynthesis and cell development. This system could be the seat of function of oncogene proteins that on mutation cause the regulatory malfunctions of cancer.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—284 939

Project Description

On the Regulation of the First Step of Protein Biosynthesis

Introduction:

A logical point of regulation for protein biosynthesis is its first step, the aminoacylation of tRNA. An important finding concerning this possibility is a complex-bound valyl-tRNA synthetase from yeast that requires a thiol for activity and is sensitive to the dithiol binding agent, arsenite [S. Black (1983) *J. Biol. Chem.* **258**, 2112-2114]. The suggestion that the enzyme's regulation involves dithiol-disulfide interchanges between intimately associated proteins is supported by a spectactular oscillatory interconversion between two physical states of this complex which is affected by oxido-reductive agents [S. Black (1986) *Science* **234**, 1111-1114]. The enzyme's activity is also markedly affected by opposed effects of saturated and unsaturated (*i.e.*, reduced and oxidized) fatty acids [S. Black (1985) *J. Biol. Chem.* **260**, 433-440]. A route to definitive characterization of the thiol-regulated process was opened by the finding that the reductive activation of the enzyme can occur in aqueous solutions of ethylene glycol. In this reagent the activation, which probably occurs normally in seconds or less, is slowed so that it can be observed for periods of 10-60 minutes, and it can thus be studied in the manner of an enzymatic reaction. In the ethylene glycol test, the enzyme sometimes oscillates between states of high and low activity, demonstrating that the activation process is reversible.

Present Status

After passage through a Sephadex G50 column the weakened enzyme gives a very large activity response to glutathione added in very high concentration (.3M). The paradox between the high activity and the high concentration requirement raises the question: Is glutathione an actual regulator of the system, a substitute for a presumably genuine regulator, or a co-factor requiring another substance that might allow it to function in physiologically reasonable amounts? New evidence indicates that another factor is involved that is at once difficult to remove from the enzyme complex and then difficult to re-associate with it. The difficulties are unquestionably due to a complicated role for -SH groups. Nevertheless, a crude preparation of the substance is now available and its nature and function can be studied. It promises to have a major function in controlling cell growth.

Publications:

Black, S: Book review of *Methods in Enzymology: Guide to Protein Purification*, Vol. 189, page 150, 1990, Deutscher, M. P., Ed., Academic Press. *Anal Biochem.*.

Patents: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 23,330-13 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Aldoheptose Biosynthesis and Its Regulation and Hepatitis Non-A, Non-B

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	William G. Coleman, Jr. Ph.D.	Research Microbiologist	LBP NIDDK
Others:	Lishi Chen, Ph.D.	Visiting Fellow	LBP NIDDK
	Li Ding, M.D.	Visiting Fellow	LBP NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER.
3.3	3.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Aldoheptose Biosynthesis. *E. coli* K-12 aldoheptose mutants, carrying the *cysE-pyrE* linked mutation *rfaD* or *rfa-2*, were previously isolated and genetically characterized. The *rfaD* phenotype includes increased permeability to a large number of hydrophobic antibiotics, in addition to altered LPS synthesis. The *rfaD* gene initially identified in the Clarke-Carbon Genomic Bank was cloned. The precise location of the *rfaD* gene on a 1.3-kilobase SspI-HpaI fragment has been determined. The *rfaD* gene and the flanking regions have been completely sequenced, and the coding and regulatory regions have been defined. The location of the *rfaD* gene on the physical map of the *Escherichia coli* chromosome has been resolved. RfaD plasmids express *in vivo* and *in vitro* a protein with the molecular weight of 37,000. The protein, ADP-L-glycero-D-mannoheptose-6-epimerase, has been purified to homogeneity and partially characterized. N-Terminal analysis of purified ADP-D-glycero-D-mannoheptose-6-epimerase confirms the first 34 amino acid sequence deduced from the nucleotide sequence of the *rfaD* gene coding region. The rfaD protein contains the fingerprint sequence of the ADP-binding βαβ-fold of NAD-binding proteins, near its N-terminus. The *rfaD* gene studies have been extended to include the clinical pathogen *Pseudomonas aeruginosa*. The second *cysE-pyrE* linked mutation, designated *rfa-2*, resulted in heptoseless LPS (chemotype Re) and increased permeability to hydrophobic and hydrophilic agents. We have cloned the *rfa-2* gene, resolved its sequence and flanking nucleotides, as well as determined its physical location on the *E. coli* chromosome. The *rfa-2* gene encodes a 36Kd protein. Interspecific complementation studies demonstrated that the *E. coli* K-12 *rfa-2* gene complements the chemotype Re LPS mutant of *S. typhimurium*, designated *rfaC*.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—[illegible] 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 23,580-28 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Mammalian Transposons

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Anthony V. Furano, M.D.	Medical Officer (Research) and Chief, Section on Genomic Structure and Function, LBP	LBP NIDDK
Others:	Karen Usdin, Ph.D.	Visiting Associate	LBP NIDDK
	Bruce E. Hayward, Ph.D.	Visiting Associate	LBP NIDDK

COOPERATING UNITS *(if any)*

Dr. Francois Catzeflis, Institute of Evolutionary Science, Montpellier, France;
Dr. Michael Seidman, Otsuka Pharmaceuticals, Rockville, MD

LAB/BRANCH
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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Members of the L1 transposon family (long interspersed repeat DNA or LINE family) of rats are 6.7 kb long, 5 kb of which is devoted to protein encoding sequence (ORFs). A 610 bp CpG-rich sequence is at the left end of the element, and a guanine-rich polypurine:polypyrimidine sequence is near the right end. Although the protein encoding sequences of mammalian L1 families are highly conserved, the promoter sequences are completely distinct. This means that these families have been independently amplified in various mammalian species. Our recent identification of a highly repeated murine ancestral L1 family indicates that L1 amplification has occurred repeatedly during mammalian evolution. Our previous demonstration that the rat L1 family contains an active regulatory region was the first evidence that L1 DNA is not just some non-functional "junk" DNA. We have further characterized the L1 regulatory region and found that it contains three regions that can form specific DNA:protein complexes with nuclear extracts as judged by gel retardation experiments. Deletion analysis shows that the first region binds a transcriptional inhibitory factor while the third region binds a stimulatory factor. We also have found that the L1 regulatory region enhances the activity of regulatory sequences that are located 5' to it. In addition to activating cryptic promoters it also strongly stimulates both the early and late SV40 promoters. We previously showed that the L1 guanine-rich polypurine:polypyrimidine sequence destabilizes contiguous duplex DNA, adopts several non-B DNA structures *in vitro*, and that the L1 non-B structures compete with target site non-B structures for supercoil energy which *in vivo* might modulate the supercoil dependent properties of L1 elements and their target sites. In examining the effects of the L1 polypurine:polypyrimidine region *in vivo*, we now find that this region decreases the replication of plasmids bearing the SV40 replicon and alters the apparent activity of certain eukaryotic promoters. In parallel studies we have shown that homoguanine stretches enhance the mutation rate of adjacent DNA sequences *in vivo*.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 23,750-05 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Bacteriophage T4 Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Deborah M. Hinton, Ph.D.	Research Chemist	LBP NIDDK
Other:	Mridula Sharma, Ph.D.	Visiting Fellow	LBP NIDDK
	Roslyn March-Amegadzie, Ph.D.	Staff Fellow	LBP, NIDDK

COOPERATING UNITS *(if any)*

None

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Laboratory of Biochemical Pharmacology

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Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.7	2.4	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐x(c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During infection by bacteriophage T4, the use of specific promoter sequences for the transcription of early, middle, and late genes correlates with different phage-induced modifications to the host polymerase. We are studying the expression of a cluster of T4 genes (5' → 3'): *X.1* (DNA endonuclease, related to intron-encoded endonucleases), *uvsX* (recombination protein), 40 (stimulates head formation), and 41 (DNA replication protein, part of the primase-helicase) as a model for understanding how the phage regulates middle RNA.

In vivo the T4 motA protein is required for transcription from T4 middle promoters. We have demonstrated that *in vitro* expression from the middle promoter located 190 bases upstream of *uvsX* is dependent on motA protein and middle polymerase (host RNA polymerase isolated from T4-infected cells at the start of middle gene expression, 4 min post-infection at 37°). RNA polymerase isolated from uninfected cells gives a minor amount of RNA from this promoter, but this synthesis is not motA-dependent. Previous studies have shown that middle polymerase differs from unmodified polymerase by the association of 2 phage proteins (10 kDa protein and 15 kDa, rbpA protein) and by the ADP-ribosylation of the α subunits. Thus, this *in vitro* transcription system provides the basis for a detailed study of the phage and host factors needed to regulate T4 middle gene expression.

The biological function of the T4 *X.1* gene is unknown since an T4 *X.1* mutant is normal for phage growth and UV repair. We have found that the X.1 open reading frame shares tremendous similarity with 4 other T4 proteins of unknown function and that together these proteins share regions of homology with endonucleases of mobile group I introns. We have also found that, like these intron-encoded endonucleases, the X.1 protein is a Mg^{++}-dependent DNA endonuclease with preferred sites for cutting. We speculate that X.1 and the 4 other T4 ORFs represent a family of endonucleases related to the endonucleases present in mobile group I introns.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,140-25 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Structure and Function of the Tryptophan Synthase Multienzyme Complex

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Edith Wilson Miles, Ph.D.	Research Chemist	LBP NIDDK
Others:	Syed A. Ahmed, Ph.D.	Senior Staff Fellow	LBP NIDDK
	Xiang-Jiao Yang,, Ph.D.	Visiting Fellow	LBP NIDDK
	Sergei Ruvinov, Ph.D.	Visiting Fellow	LBP NIDDK
	Zichun Lu, Ph.D.	Visiting Fellow	LBP NIDDK

COOPERATING UNITS (if any) Drs. D.R. Davies & C.C. Hyde, LMB, NIDDK; P. McPhie, LBM, NIDDK; A. Ginsburg, LB,NHLBI; P. Brzovic & M.F. Dunn, Univ. of California, Riverside; A. Mozzarelli & G.L. Rossi, Univ. of Parma, Italy; K. Yutani, Osaka U., Japan; K.S. Anderson, Yale Univ., New Haven, CT; & K.A. Johnson, Pennsylvania State Univ., University Park, PA

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Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER.
4.6	4.3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The tryptophan synthase $\alpha_2\beta_2$ multienzyme complex is an excellent model system for investigating enzyme mechanism, protein-protein interaction, metabolite channeling, and ligand-dependent site-site interactions. Our recent determination by x-ray crystallography of the three-dimensional structure of the tryptophan synthase complex $\alpha_2\beta_2$ from *Salmonella typhimurium* provides a useful framework for further exploration of the relationships between protein structure and protein function. We are using the three-dimensional structure to select enzyme residues for site-directed mutagenesis. Our studies with wild type and mutant forms have led to the following findings during the last year: (1) Single turnover quench-flow kinetic studies demonstrate that indole is a channeled intermediate and L-serine modulates the rate of indole formation. (2) Certain mutations alter the kinetics of indole formation and the rate of passage of indole through the channel. (3) Alteration of a flexible loop in the α subunit by mutagenesis or by proteolytic cleavage modifies allosteric effects and the reciprocal communication between subunits. (4) Certain mutations in the active site and tunnel region of the β subunit alter the reaction and substrate specificity and lead to substrate-induced inactivation. (5) Scanning microcalorimetric and circular dichroism studies of the thermal unfolding of wild type and mutant forms of tryptophan synthase complex and of its subunits reveal effects of subunit interaction and of single amino acid residues on protein stability. (6) Spectroscopic studies of chromophoric intermediates formed between pyridoxal phosphate and substrates at the active site of the β subunit demonstrate that the equilibrium distribution of intermediates is affected by pH, by ligands of the α subunit, and by mutation.

U.S. GOVERNMENT PRINTING OFFICE 1990 0—264 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,150-20 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Noncovalent Intermolecular Interactions in Biochemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Allen P. Minton, Ph.D.	Research Chemist	LBP NIDDK
Others:	German Rivas, Ph.D.	Visiting Fellow	LBP NIDDK
	Saleh Darawshe, Ph.D.	Visiting Fellow	LBP NIDDK
	Geoffrey Howlett, Ph.D.	Courtesy Associate	LBP NIDDK

COOPERATING UNITS *(if any)*

A. Furst, L. Holladay, Beckman Instruments, Palo Alto, CA

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.9	2.6	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The interaction between purified platelet fibrinogen receptor and fibrinogen in the presence and absence of calcium and/or solubilizing detergents has been studied by analytical centrifugation.

A mathematical model for the time course of separation of macromolecules on self-forming cesium chloride density gradients has been developed.

A statistical-thermodynamic model has been developed for the effect of excluded volume due to fibrous elements of the cytomatrix upon macromolecular equilibria in the fluid phase of cytoplasm.

U.S. GOVERNMENT PRINTING OFFICE 199 0—564 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,260-25 LBP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Nancy G. Nossal	Research Chemist and Chief, Section on Nucleic Acid Biochemistry. LBP	LBP	NIDDK
Others:	Peter Spacciapoli, Ph.D	IRTA Fellow	LBP	NIDDK
	Lisa Hobbs, Ph.D	IRTA Fellow	LBP	NIDDK
	Todd Capson, Ph.D	Senior Staff Fellow	LBP	NIDDK

COOPERATING UNITS *(if any)*

Dr. Stephen Benkovic, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS. 2.9	PROFESSIONAL. 2.6	OTHER. 0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are continuing our study of the *E. Coli* bacteriophage T4 model system for duplex DNA replication in which efficient DNA replication *in vitro* is achieved with purified proteins encoded by T4 phage: these include T4 DNA polymerase (gene 43), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, the genes 41, 61, and 59 primase-helicase, RNase H, and DNA ligase.

Assembly of the polymerase and accessory proteins on the primer template. Using a system in which a photoactivatable aryl azide can be moved stepwise through a thirty base region of the primer, we find that the accessory proteins and 32 protein can be cross-linked to the primer in the presence of a non-hydrolyzable ATP analogue. The initial binding of the three accessory proteins and ATP to a 32 protein-covered primer-template is followed by ATP hydrolysis, binding of polymerase, and movement of each of the accessory proteins to yield a complex capable of processive DNA synthesis.

Protein-protein interactions required for strand displacement synthesis. Mutations in the T4 DNA polymerase can decrease its ability to interact with the proteins that allow strand displacement synthesis with the wild type enzyme. The purified *ts*L141 mutant polymerase (alanine changed to valine at 737) is stimulated by the accessory proteins and 32 protein, but is arrested at pause sites on single-stranded templates more frequently that the wild type, and carries out practically no strand displacement synthesis on a forked DNA template with the accessory proteins, 32 protein, and the 41 protein helicase. We have recently cloned and purified to homogenity the T4 gene 59 protein, which increases DNA unwinding by the 41 helicase. We find that addition of this 59 protein is absolutely required for strand displacement synthesis with the *ts*L141 mutant polymerase. The position of the *ts*L141 mutation (737) and that of a second site mutation (771) that suppresses its phenotype are C-terminal to the regions where T4 polymerase shares homology with eukaryotic DNA polymerases, and may be in a region important for its interactions with the other T4 replication proteins.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—[illegible] 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,590-21 LBP

PERIOD COVERED
October 1, 1990, through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structure and Interactions of Biologically Important Macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Harry A. Saroff, Ph.D.	Research Chemist (Intermittent)	LBP NIDDK
Other:	Elemer Mihalyi, M.D., Ph.D.	Special Volunteer	LBP NIDDK

COOPERATING UNITS (if any)

National Center for Drugs and Biologics

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.8	1.5	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and similarity of these sequences. The property of uniqueness (the occurrence of a *small* peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under continued investigation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,709-10 LBP

PERIOD COVERED

October 1, 1990, through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Polyamine Biosynthesis and Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Celia White Tabor, M.D.	Medical Officer (Research)	LBP NIDDK
Others:	Herbert Tabor, M.D.	Supervisory Medical Officer (Research); Chief, Section on Pharmacology, LBP; and Chief, Laboratory of Biochemical Pharmacology	LBP NIDDK
	Qiao-Wen Xie, Ph.D.	Visiting Associate	LBP NIDDK
	David Balasundaram, Ph.D.	Visiting Fellow	LBP NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.8	3.3	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Polyamines (putrescine, spermidine, and spermine) are major cellular components and have been shown to be involved in many systems related to growth and differentiation. Our current and older studies have been directed at learning how these polyamines are synthesized and regulated, and their physiological function. We have: (1) established the pathways for the biosynthesis of these amines in prokaryotes and eukaryotes and isolated the enzymes for the various steps in the pathways; (2) identified the genes responsible for each of the biosynthetic steps and constructed mutants with deletions in the various genes; (3) constructed plasmids that contain these genes and used the strains containing these plasmids to overproduce the encoded enzymes; (4) used the amine-deficient mutants to study the physiological effects of polyamine deprivation; (5) sequenced the gene coding for *S*-adenosylmethionine decarboxylase in both *E. coli* and *S. cerevisiae* and the gene coding for spermidine synthase in *E. coli;* (6) demonstrated that *S*-adenosylmethionine decarboxylase is first formed as a proenzyme in both *E. coli* and yeast and is cleaved post-translationally with the conversion of serine to a covalently-bound pyruvoyl group that is essential for activity; and (7) studied the effect of site-specific mutagenesis on the conversion of the proenzyme to the active enzyme. The most recent work involves the construction of null mutants in both *Escherichia coli* and in *Saccharomyces cerevisiae*, and studies on the physiological effects of the resultant complete absence of spermidine and spermine. The most striking results were found with null mutants of *Saccharomyces cerevisiae;* these mutants have an absolute requirement for spermidine and/or spermine, and show very striking morphological changes after depletion of these amines. Depletion of spermidine and spermine in *S. cerevisiae* also results in a marked irreversible defect in mitochondrial structure and function.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—264 933

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 24,940-18 LBP

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Yeast RNA Virology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Reed B. Wickner, M.D.	Medical Director, USPHS and Chief, Section on Genetics of Simple Eukaryotes, LBP	LBP NIDDK
Others:	Tsutomu Fujimura, Ph.D	Visiting Scientist	LBP NIDDK
	Yutaka Matsumoto, Ph.D	Staff Fellow	LBP NIDDK
	Jonathan D. Dinman, Ph.D	IRTA Fellow	LBP NIDDK
	William R. Widner, Ph.D	IRTA Fellow	LBP NIDDK
	Rosaura P.C. Valle, Ph.D	Visiting Fellow	LBP NIDDK
	Yang-Ja Lee, Ph.D	Special Volunteer	LBP NIDDK
	Juan Carlos Tercero, Ph.D	Special Volunteer	LBP NIDDK
	Juan Carlos Ribas, Ph.D	Special Volunteer	LBP NIDDK

COOPERATING UNITS (if any)
Richard Fishel, Laboratory of Chromosome Biology, BRI--Basic Research Program, FCRF, Frederick, MD 21701.

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Genetics of Simple Eukaryotes

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
9.4	9.0	0.4

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

We have shown that the L-A dsRNA virus of *Saccharomyces cerevisiae* has, in its (+) strand, two open reading frames: ORF1, encoding the major coat protein (*gag*) and ORF2, encoding the viral RNA-dependent RNA polymerase and a single-stranded RNA binding activity (*pol*). We found that *pol* is expressed only as a *gag-pol* fusion protein, produced by a -1 ribosomal frameshift mechanism indistinguishable from that used by retroviruses to make their *gag-pol* fusion proteins. We developed frameshift vectors which are being used to screen for drugs affecting ribosomal frameshifting, a new approach to searching for anti-retroviral drugs. Either increasing or decreasing the efficiency of frameshifting adversely affects replication by the L-A proteins, presumably because of the altered ratio of *gag-pol* to *gag* proteins, and we isolated chromosomal mutants that dramatically increase the efficiency of frameshifting. We defined the domains essential for viral replication around the RNA polymerase consensus sequence in *pol*, anddelimited the location of the single-stranded RNA binding domain. In the replication reaction (*in vitro* synthesis of (-) strands on a (+) strand template) the polymerase first binds at an internal site on the template molecule (*the internal replication enhancer*) and then attaches to the 3' end where it then begins synthesis (a looping mechanism).

We cloned and sequenced the *SKI2* and *SKI8* genes, members of the yeast intracellular antiviral system that limits replication of the apparently unrelated dsRNA viruses, L-A and L-BC and the 20S RNA circular single-stranded RNA replicon. *SKI2* has homology with RNA helicases and ribonucleoproteins, while *SKI8* has a repeat sequence first described in β-transducin, but also found in other proteins. Our studies of the *MAK10* gene show that it is repressed by glucose, and, unexpectedly, its control sequences are inside the coding region of an adjacent gene. *MAK*3 encodes an N-acetyltransferase necessary for acetylation of the N-terminus of the L-A major coat protein.

The 20S RNA circular single-stranded replicon is apparently naked in the cell, since we found that what had been reported by others to be its coat was actually a heat-shock protein adventitiously co-purifying with the RNA. We showed that 20S RNA encodes a 90 kDa protein that is expressed in infected yeast cells and has some homology to RNA polymerases of (+) strand RNA viruses.

Annual Reports of the Laboratory of Chemical Biology
National Institute of Diabetes and Digestive and Kidney Diseases

The Laboratory of Chemical Biology conducts research on molecular biology and genetics, especially as related to genetic diseases, and on the structure, function and dynamics of proteins. A major emphasis of the Laboratory is in understanding the molecular processes involved in the developmental control of the expression of the human hemoglobin genes. As part of this work, extensive studies on the pathophysiology of genetic diseases of hemoglobin and new approaches to their treatment have been developed. A second major emphasis of the Laboratory is in the study of forces that stabilize globular proteins. A third program concentrates on cytogenetic analysis of patients with genetic illnesses. Other new research initiatives include the cloning and characterization of the human erythropoietin receptor gene and the development of a transgenic mouse model of sickle cell disease. The protocol for treatment of sickle cell disease patients with hydroxyurea to elevate fetal hemoglobin has been enlarged to include protocols with the addition of recombinant human erythropoietin and also the treatment of patients with the thalassemia syndromes. A new program to establish a facility for the production of transgenic mice has been initiated.

The Laboratory has three Sections. The Section of Protein Chemistry and Conformation, under Dr. Hiroshi Taniuchi, is devoted primarily to studying the folding and assembly of globular proteins, especially cytochrome c. The Section on Molecular Forces and Assembly is the home of the Cytogenetics Unit under Dr. Beverly White, which is a joint endeavor of the Inter-Institute Genetics Program of the Clinical Center and NIDDK. The Section on Molecular Biology and Genetics, under Dr. Alan N. Schechter, is concerned primarily with the molecular basis of the developmental control of gene expression, especially in human erythroid cells, and its relevance to the understanding of the molecular basis of disease states and possible approaches to their therapy. A new Unit, on Molecular Hematology, under Dr. Griffin Rodgers, has been established in that Section.

During this last year, several personnel changes have occurred. Dr. Griffin P. Rodgers, who recently received scientific tenure, has been appointed head of the new Unit on Molecular Hematology. Dr. Betty Peters has begun work under her new status in the Robert Wood Johnson Foundation Minority Medical Faculty Development Program. Dr. Patricia Berg has left to accept a faculty appointment in the Division of Pediatric Hematology/Oncology at the University of Maryland School of Medicine. Dr. Yu-can Du of the Shanghai Institute of Biochemistry and Dr. Eitan Fibach of the Hadassah Medical School in Jerusalem Israel have worked in the Laboratory for extended periods under the Courtesy Associate Program for senior scientific visitors. Dr. Kyung Bae of the Genetic Engineering Research Institute in Taejon, Korea and Dr. Suthat Fucharon of the Maidol Medical School in Bangkok, Thailand have worked in this Laboratory under sponsorship of the World Health Organization. Dr. C. B. Anfinsen continues as a Scientist Emeritus in this Laboratory and is a frequent visitor.

Extensive research collaborations exist within this Laboratory and with other Laboratories in this Institute, in NIH, and nationally and internationally as outlined in the individual Research Project Reports. A formal collaboration has been established with the Clinical Center's Inter-Institute Medical Genetics Program to fund a clinical and research cytogenetic program. Clinical collaborations also exist with the Clinical Hematology

Branch of NHLBI and other units. It is under the aegis of this collaboration that the clinical studies of hydroxyurea treatment of sickle cell patients is done. In addition, a formal collaboration has been established involving the exchange of personnel and resources with Dr. David Hankins of George Mason University, Fairfax, Virginia and the Division of Hematology and Oncology of the Children's National Medical Center in Washington, D.C.. The participation of this Laboratory in the NIH Inter-Institute Medical Genetics Program and the NIH-George Washington University Hematology Training Program continues to grow. Other collaborations include those with the Department of Pediatrics of Johns Hopkins Medical School, the Department of Medicine at the University of Mississippi School of Medicine, the Department of Medicine at George Washington University School of Medicine, the Howard University Sickle Cell Center; the INSERM Unit 15 in Paris, France; the Hasharon Hospital and the Hadassah Medical School in Israel; the Department of Physiology of the Nippon Medical School in Tokyo, Japan; and the Medical Genetics Institute of the Children's Hospital of Shanghai, China.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 25008-28 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
The Core Loop Interaction That Controls Protein Folding

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Hiroshi Taniuchi	Chief, Section on Protein Chemistry and Conformation	LCB, NIDDK
Others:	Alice Fisher	Chemist	LCB, NIDDK
	Greg Charles	Biological Aid	LCB, NIDDK

COOPERATING UNITS (*if any*)

LAB/BRANCH	Laboratory of Chemical Biology
SECTION	Section on Protein Chemistry and Conformation
INSTITUTE AND LOCATION	NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

This project has been combined with project Z01 DK 25011-17 LCB.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE. 1981 0-864-438

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25011-17 LCB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Protein Folding Problem

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hiroshi Taniuchi	Chief, Section on Protein Chemistry and Conformation	LCB, NIDDK
Others:	Alice Fisher	Chemist	LCB, NIDDK
	Greg Charles	Biological Aid	LCB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 2.15	PROFESSIONAL: 1.7	OTHER: 0.45

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A long range goal of this project is to find a way to predict the three-dimensional structure of proteins on the basis of the amino acid sequences. In recent years and this year, we have made several key findings which have unified all previous studies of protein folding in this Section. Two manuscripts containing a mass of data have been completed by A. Fisher and H. Taniuchi in this year. In the following the findings and principles written in these manuscripts are summarized.

A large number of homologous and hybrid 2-fragment complex of 4 different types were prepared from appropriate heme and apofragments or apoproteins of horse, tuna, yeast iso-1 and Candida cytochromes c. The complexes were characterized for structure, stability and Gibbs energy change for binding. The results have allowed us to assign 4 folding units which collectively represents cytochrome c folding. Unit 1 folds by itself and consists essentially of the right channel hydrophobic core and a part of heme. R. E. Dickerson and colleagues have found that the COOH-terminal helix contacts with the NH2-terminal helix to form a right channel. Units 2,3 and 4, respectively are assigned to the cores on the left and right sides and at the bottom of heme.

Based on evidence of the folding unit - unit interaction we propose that eukaryotic cytochrome c folds in two alternative ways depending on the temperature after attachment of heme to apoprotein by action of cytochrome c synthetase: Unit 1 ->3->2->4 or 1->2->3->4. Gibbs energy change for binding was found to vary depending on which species of heme fragment binds with which species of apofragment or apoprotein. This is more pronounced going from the less ordered complex to the more ordered. Evidence suggests that such Gibbs energy change difference of complexes containing the Fe-S bond is mainly due to perturbation of the folding unit-unit interaction, which may be mediated by contacting core groups. Based on this, a self-consistent set of residue substitutions to account for this Gibbs energy difference is assigned. Analysis of Gibbs energy change difference based on this assignment has suggested that a network of core group interaction may expand throughout the folding unit cores from Unit 1 as folding progresses. A model of this extra core interaction, core loop interaction/core loop coalescence which drives folding is proposed.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-439

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25016-18 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Trans-Acting Factor(s) Controlling Globin Genes in K562 Cells and Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Alan N. Schechter	Chief	LCB, NIDDK
Others:	Frank Shafer	NRSA Fellow	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Zi Yao Liu	Visiting Associate	LCB, NIDDK

COOPERATING UNITS *(if any)*
DMNB, NINDS (Drs. S. Karlsson and H. Dave); Department of Pathology, USUHS, Bethesda, MD (Dr. Pablo Gutman); Connaught Laboratories, Toronto, Canada (Dr. S. X. Cao); University of South Carolina, Columbia, SC (Dr. M. Dewey); Petah Tikva Hospital, Israel (Dr. M. Mittelman)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

K562 is an erythroleukemic cell line used as a model for the study of the control of human globin gene expression. These cells do not support transcription of the beta-globin gene but do express transcripts of epsilon- and gamma-globin genes at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence of trans-acting factors which exert their action on sequences corresponding to the promoters of these genes.

We have previously reported the presence of a transcriptional control element with properties of a silencer extending from -392 to -177 bp relative to the cap site of the human epsilon-globin gene. We also showed that this silencer has stronger inhibitory activity in HeLa cells than K562 human erythroleukemia cells. Using deletion mutants and synthetic oligonucleotides in transient expression assays, DNA sequences responsible for this effect have been further delimited to 44 nucleotides located between -294 and -251 bp. Gel electrophoresis mobility shift assays and DNaseI footprinting assays demonstrate that these negative regulatory sequences are recognized differently by proteins present in nuclear extracts obtained from HeLa and K562 cells. The protein present in K562 cells, but not in HeLa cells, that interacts specifically with this silencer binds to the same sequence recognized by the yeast binding protein ABF1. Possible mechanisms by which these proteins may regulate epsilon-globin gene transcription in erythroid and non-erythroid cells are discussed.

We have constructed several mice using transgenic techniques that express both the human alpha-globin and beta-S-globin polypeptides at levels of 15-20% of endogenous mouse hemoglobin. Data on erythrocyte functional properties now being studied include morphological analysis oxygen affinity curves, hematological parameters and rheology. Further cross breeding studies should result in a line of transgenic animals manifesting the sickle cell phenotype.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-438

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25021-16 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Sickle Cell Anemia: The Intracellular Polymerization of HbS

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Constance Tom Noguchi	Research Physicist	LCB, NIDDK
Others:	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
LCDB , NIDDK (J. Blanchette-Mackie); George Washington University (Dr. L. Lessin), Howard University (Dr. W. Poillon), University of Mississippi School of Medicine, Jackson, MS (Dr. M. H. Steinberg)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION
NIDDK, Bethesda, MD

TOTAL MAN-YEARS: 0.3	PROFESSIONAL: 0.3	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Polymerization of sickle hemoglobin is determined by oxygen saturation, hemoglobin concentration and hemoglobin composition. Within the erythrocyte, hemoglobin polymerization can lead to abnormal rheology. A detailed analysis and understanding of the effect of intracellular polymerization on disease manifestation will clarify the cellular basis of disease in the sickle syndromes.

Defect in urine concentrating ability is a frequent finding in individuals with sickle cell trait. The mechanism is unclear, but may result from intracellular polymerization of sickle hemoglobin in erythrocytes, leading to microvascular occusion in the vasa recta of the renal medulla. If such is the case, the severity of the concentrating defect should be determined by the percentage of sickle hemoglobin present in erythrocytes. To test this hypothesis, urinary concentrating ability was examined following overnight water deprivation and intranasal dDAVP in 27 individuals with sickle trait who had a normal alpha-globin genotype (aa/aa), or were either heterozygous (-a/aa), or homozygous (-a/-a) for gene deletion alpha thalassemia, since alpha-thalassemia modulates the sickle hemoglobin concentration in sickle trait. The urinary concentrating ability was less in the aa/aa genotype than in the -a/aa or -a/-a genotypes. Following dDAVP, the urine osmolality was greater in patients with the -a/-a genotype than the -a/a genotype (822 vs 672 mOsm/kg H20); patients with the -a/aa genotype had greater concentrating ability than individuals with a normal a-globin gene arrangement. A linear correlation also existed for urine concentrating ability and polymerization tendencies calculated for an oxygen saturation of 0.4. We conclude that the severity of hyposthenuria in sickle trait in heterogeneous. It is determined by the amount of sickle hemoglobin polymer that in turn is dependent upon the percent of sickle hemoglobin, which is itself related to the a-globin genotype.

Recently, we have initiated studies to examine the effect of fetal hemoglobin on the solubility of deoxyhemoglobin S under physiological conditions and of the effect of polymer formation on the rheological properties of the sickle erythrocyte. Both of these analyses will further understand the relationship between polymer formation and disease severity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25025-15 LCB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Mechanism of Antigen-Antibody Interaction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hiroshi Taniuchi	Chief, Section on Protein Chemistry and Conformation	LCB, NIDDK
Others:	Paola Rizzo	Visiting Fellow	LCB, NIDDK
	Caterina Tinello	Visiting Fellow	LCB, NIDDK
	Emmanuel Wong	Biological Aid	LCB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 2.55	PROFESSIONAL: 2.3	OTHER: 0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structures of several Fab fragment-protein antigen complexes, shown by X-ray crystallography by others, have revealed the details of group contact in the antigen-antibody interface. However, despite such elucidation of the interacting groups, the antigen binding-interaction and the interaction mechanism responsible for the fine specificity are not well understood. For example, a combination of hydrophobic interaction, hydrogen bonds, electrostatic interaction and Van der Waals force is considered sufficient to describe the antigen-antibody interaction.

In the studies of protein folding (see Project Z01DK25011-17 LCB) we have developed a model of a new non-covalent interaction, namely core loop interaction/core loop coalescence which drives folding. This core loop interaction is assumed to be sensitive to the detail of group contact by which the interaction is mediated. We have thought that such core loop interaction may be responsible for the fine specificity of antigen-antibody interaction. If this hypothesis is correct, antigen binding to antibody should stabilize the hydrophobic cores far from the antigen-binding site of antibodies. To test this hypothesis, we have measured amide hydrogen exchange rates of monoclonals 4-128-6, 4-74-6 and 2-96-12 to yeast iso-1-cytochrome c in the presence and absence of antigen. The amide hydrogen-deuterium exchange was followed by infrared spectrophotometry at pD 7.0, 24-25°C. Approximately 48 and 43 more mol of amide hydrogens per mol of antigen binding site, respectively of monoclonals 4-128-6 and 4-74-6 remained unexchanged after 48 and 72 h in the presence of antigen than in the presence of horse cytochrome c. Horse cytochrome c does not crossreact with these monoclonals.

The results with monoclonal 2-96-12 has pointed to a novel aspect: both yeast iso-1 and tuna cytochromes c which have higher affinity to the monoclonal have decreased the hydrogen exchange rate more than horse cytochrome c which has lower affinity. These observations indicate that the core regions removed from the antigen-binding site of antibodies are stabilized by antigen-binding. In light of the core loop interaction theory it follows that the hydrophobic cores of V_H and V_L domains may influence the antigen-antibody interaction.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 438

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25028-13 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Development of Non-Invasive Methods to Assess Sickle Cell Patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Griffin P. Rodgers	Chief, Molecular Hematology Unit	LCB, NIDDK
Others:	Hiroyuki Hiruma	Visiting Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
Clinical Hematology Branch, NHLBI (A. W. Nienhuis); Transfusion Medicine, CC (H. Klein); BEIB (Eli Walker); Biometry Branch, NEI (M. Podgor); MRC Laboratory, Kingston, Jamaica (G. Serjeant); Nippon Medical School (Dr. N. Uyesaka)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Unit on Molecular Hematology

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The molecular and cellular pathophysiology of the sickle cell syndromes are now appreciated with a great deal of precision. On the other hand, our understanding of the relationship of these subcellular events to the variable clinical expression of sickle cell disease remains largely speculative. A major effort of our research group has been to develop quantitative ways to clarify disease pathogenesis, as well as to assess severity and progression. Using calibrated phthalate ester separation method, which we previously described, we have now defined at least three cellular processes contributing to the extensive red cell heterogeneity that is commonly observed in the sickle cell syndromes. Using a sensitive nickel mesh filtration system, we are systematically determining the effects of changes in oxygen saturation, corpuscular hemoglobin concentration/composition and temperature of the flow properties of sickle hemoglobin containing erythrocytes. Ophthalmological studies of the patients show highly significant correlations between the extent of erythrocyte heterogeneity with conjunctival and retinal vessel pathology. As predicted by biophysical studies of polymer formation, we find that treatment of steady state sickle cell patients with selective arteriolar vasodilators results in a significant resolution of both acute conjunctival and retinal abnormalities, as well as an improvement in color vision performance. These beneficial effects occurred in the absence of a direct drug-induced inhibition of polymer formation, and therefore suggests that inappropriate vasospasm or frank vasoconstriction, perhaps in response to the altered rheology of red cell containing polymerized sickle hemoglobin is a significant contributing factor to the pathogenesis of sickle cell disease. This conclusion is also supported by our recent observation that "relative"hypertension is a significant risk factor for the occurrence of stroke in sickle cell patients. Using the technique of laser-Doppler velocimetry, and phlethysmography we have found that forearm cutaneous microcirculatory flow in sickle cell individuals show characteristic patterns, which may become "normalized" depending upon the fraction of non-S hemoglobins and during crisis. We hope that these cellular and physiological measurements will allow us to understand better the extreme spectrum of disease manifestations.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-854-838

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25045-08 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Beta Globin Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Patricia Berg	Expert	LCB, NIDDK
Others:	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
Hopital Robert Debre, Paris, France (Dr.. J. Elion and Dr. C. Lapermeroulie)
Institut National de la Sante et de la Recherche Medical Unit 15, Paris, France (Dr. D. Labie)
Children's Hospital, Oakland Research Institute, Oakland, CA (Dr. S. Perrine)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 1.2	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Regulation of the beta globin gene is important for better understanding hemoglobin disorders such as sickle cell anemia and beta thalassemia, yet this regulation is poorly understood. We are currently studying the effect of cis acting DNA sequences and trans acting proteins on beta globin gene expression to begin to approach this question.

The area upstream of the beta globin gene can be subdivided into several regions. The first 100 bp, or proximal promoter, is known to contain sequences important for both the correct initiation of transcription and for the amount of mRNA synthesized. The next 100 bp, from -100 to -200, is called the distal promoter because it seems to contain sequences required for erythroid specific transcription of the gene. We have found, by deletion analysis, that DNA between -233 and -185 is necessary for gene expression. In addition, we have defined two regions that act as silencers or negative regulatory elements, one between -610 and -490 bp, the other between -338 and -233 bp. We have also identified a protein (BP1) which binds to both silencers, making it a candidate for a repressor protein.

BP1 binds to one silencer at -530 bp, which is also the site of a mutation found in several independent silent carriers of beta thalassemia. We looked at the binding of BP1 to the mutant DNA and found that it binds more tightly than to the reference sequence, consistent with the hypothesis that BP1 is a repressor since these patients make less beta protein than normal. In addition, there are five well characterized haplotypes associated with sickle cell anemia and each one has a different DNA sequence at -530 bp. We have now analyzed the binding of BP1 to each of the haplotypes' DNA and discovered that there is a hierarchy of binding strengths which correlates with what is known about the amount of HbS present for the haplotype. For example, the DNA of the Indian haplotype binds BP1 the tightest, these patients make less beta sickle protein than usual, and exhibit clinically mild symptoms. On the other hand, BP1 binds the most weakly to DNA of the Bantu haplotype and these patients exhibit more severe symptoms than those with the Indian haplotype. Thus binding of BP1 may be a predictor, along with other factors such as the alpha gene status and level of fetal hemoglobin, of the severity of sickle cell anemia.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25058-06 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Laboratory Model of Adult Globin Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Griffin P. Rodgers	Chief, Molecular Hematology Unit	LCB, NIDDK
Others:	Arielle Boulet	Guest Worker	LCB, NIDDK
	Patricia Berg	Expert	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*

MRC Unit, University of West Indies, Kingston, Jamaica (Dr. G. Serjeant); McMaster University, (Dr. David Chui)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.3	PROFESSIONAL: 0.3	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are investigating the molecular mechanisms which control the individual and total concentrations of hemoglobins in human erythrocytes. The study of the control of hemoglobin levels has direct relevance to various hemoglobinopathies, especially thalassemia and sickle cell disease. For our experimental system, we are using the K562 human leukemic cell line, as well as peripheral blood from individuals with sickle cell disease. We are studying the effects of short-term and long-term exposure of these cells to 5-azacytidine and hemin or their phenotype and the factors that control globin gene transcription. Adult beta-mRNA expression remains undetectable, yet we have found a constitutive level of another adult type hemoglobin, delta-mRNA, whose expression is inducible both with hemin and 5-azacytidine. Because of the close sequence homologies between the delta- and beta-globin genes, experiments are underway to examine whether changes in the delta-promoter sequence may alter important protein binding sites and thereby result in the low levels of delta-globin gene expression. A 500 bp region 5' to the delta globin CAP site has been cloned into a chloramphenicol acetyl transferase (CAT) reporter plasmid. Preliminary studies demonstrate that this reporter system when transfected into K562 give quantitatively similar results (when compared to beta-CAT and epsilon-CAT) as were obtained using S-1 nuclease mapping. Identification of these putative proteins(s) binding sites may not only provide important information on the regulation of the minor hemoglobin synthesis but may allow for the characterization of trans-acting factor(s) responsible for beta-globin gene expression. Development of this functional assay system utilizing the various CAT constructions may facilitate such identification. Investigation are also currently underway to determine the effects of copper and thyroid hormone, which have been recognized clinically to modulate HbA2 levels in patients.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-438

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25059-06 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Trans-activating Factors and Globin Gene Expression: A Direct Approacch

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Harish Dave	Visiting Fellow	LCB, NIDDK
Others:	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Chemial Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Project Z01 DK 25016-18 LCB.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-938

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25060-06 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Epsilon Gene Silencer: Characterization of Trans-acting Factor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Yuko Wada	Visiting Associate	LCB, NIDDK
Others:	Betty Peters	Guest Worker	LCB, NIDDK
	Constance Tom Noguchi	Research Physicist	LCB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 1.2	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

K562 human erythroleukemia cells constitutively express epsilon and gamma, but not beta-globin genes. The different expression of globin genes observed in intact K562 cells could be simulated in vitro as K562 nuclear extract (NE) actively transcribes the epsilon and gamma-globin gene DNA templates, but not the beta-globin gene. We have used the K562 in vitro transcription system to examine a transcriptional control element between -392 and -177bp 5' of the canonical cap site for the epsilon-globin gene which has been reported to function as a silencer. We find that K562 NE can not actively synthesize RNA in vitro from the epsilon-globin gene DNA deletion templates which contain the entire or a part of the silencer sequence but no positive regulatory region. Separating the K562 NE by ion exchange chromatography, we isolated a transcriptionally active fraction (F175) for globin genes and a fraction (F50) which contains the trans-acting factors associated with the silencer activity. The F175 was transcriptionally active for all tested globin genes including the epsilon-globin gene containing the silencer sequence. In contrast, F50 showed a strong dose dependent inhibitory effect on epsilon-globin gene transcription directed by either unfractionated K562 NE or F175. This suppression by F50 was not observed on transcription of adenovirus 2 major late promoter. In electrophoretic mobility shift assays using the epsilon-globin gene silencer region as probe, F50 and F175 exhibited different DNA binding protein patterns: a specific protein band in F50 appears to be associated with the silencer activity. These studies suggest that this protein may be specifically responsible for the activity of the silencer element of the epsilon-globin gene and that the expression and silencing of the epsilon-globin gene during development may be modulated by the interactions of this protein with the cis-acting DNA silencer.

U.S. GOVERNMENT PRINTING OFFICE : 1991 0-[illegible]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25061-06 LCB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Human Erythropoietin Receptor Gene: Cloning and Transcription Analysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Constance Tom Noguchi	Research Physicist	LCB, NIDDK
Others:	Yuko Wada	Visiting Fellow	LCB, NIDDK
	Naoko Oda	Visiting Fellow	LCB, NIDDK
	Zi-Yao Liu	Visiting Associate	LCB, NIDDK
	Kyung Chin	Research Biologist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*

George Mason University (Dr. W. D. Hankins), Korean Collection for Type Cultures, Korean Institute of Science of Technology, Daejon, Korea (Dr. K. Bae), Nippon University, Tokyo, Japan (Dr. Y. Wada), Peking Union Medical College Hospital, Beijing, The People's Republic of China (Dr. Y. Wu)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.8	PROFESSIONAL: 0.8	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Erythropoietin (EPO) stimulates the proliferation and differentiation of erythroid cells and is believed to be the primary regulator of erythropoiesis. The action of erythropoietin is initiated by its binding to a specific cell surface receptor followed by receptor-mediated endocytosis. However, the molecular details regarding the manner in which erythropoietin binding to its receptor stimulates erythropoiesis remains unknown. We have isolated, characterized and sequenced a genomic clone of the human erythropoietin receptor from a placental genomic library. The coding region spans about 6.5kb with seven intervening sequences ranging in size from 81 bp to 2.1 kb.

The human EPO receptor contains a palindromic sequence 5' of the translated region which consists of an almost perfect inverted repeat of 12 nucleotides (CAGCTGC(G/C)TCCG) centered about G at -92 from the first codon. An inverted SP1 binding site (CCGCCC) and an inverted GATA-1 binding site (TTATCT) are located at positions -151 and -179, respectively, and CACCC sequences are located at -585 and further upstream. No TATA or CAAT sequences are in this 5' flanking region. However, this region as far as -275 has a 72% GC content compared with an overall GC content of 56%.

A 1Kb Bam H1 fragment of the human EPO receptor containing 700 bp of sequences 5' of the coding region was transcribed in an in vitro transcription assay; initiation of transcription appeared to be around 132 just down stream from the inverted SP1 site at -151. T1 analysis of human EPO receptor mRNA also maps the site of transcription initiation to this region. Within 180 nucleotides 5' to the first exon are three regions with 70% greater homology with the murine EPO receptor.

We are currently in the process of detailed analysis of promoter elements and other cis-acting sequences which may provide information regarding possible trans-acting factors which regulate the developmental expression of the EPO receptor and more generally the developmental response to erythropoietin. The study of this gene including its similarities with other hematopoietic cytokine receptors should help elucidate aspects of the transcriptional and possible translational control of the EPO receptor in human erythroid cells and thus its role in signal transduction and erythroid differentiation. We have also established several transgenic mice expressing the EPO receptor and will examine the effects on the animal's phenotype.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25063-05 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of Hydroxyurea on Fetal Hemoglobin Synthesis in Congenital Hemoglobin Disorders

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Griffin P. Rodgers	Chief, Molecular Hematology Unit	LCB, NIDDK
Others:	Shu-zhen Huang	Visiting Associate	LCB, NIDDK
	Hiroyuki Hiruma	Visiting Fellow	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
CHB, NHLBI (A.W. Nienhuis); CB, NEI (Dr. M. Roy); BEIB (Mr. E. Walker); Depts. of Medicine, Pediatrics & Pathology, Johns Hopkins University, Baltimore, MD (Dr. G. Dover); Laboratory of Medical Genetics, Shanghai's Children's Hospital, Shanghai, China (Drs. Y. Zeng and S. Huang)

LAB/BRANCH Laboratory of Chemial Biology

SECTION Unit on Molecular Hematology

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.4
PROFESSIONAL: 0.4
OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our group has focused on pharmacologic manipulation of fetal hemoglobin levels in patients with genetic defects of hemoglobin. We have previously shown that hydroxyurea treatment of severely affected sickle cell patients results in about a 70 to 75% response rate defined by at least a two fold increase in F-reticulocytes and a concommitant two fold rise in per cent in hemoglobin F. Among the responders, hemoglobin F levels increased two to ten fold, generally after a lag period of about 40 to 50 days. Three patients achieved levels of fetal hemoglobin of 10 to 15%. There were no biochemical, cellular or molecular predictive factors which were associated with response to hydroxyurea. In an effort to achieve higher levels of hemoglobin F, in a more pancellular distribution, we have recently treated four patients with sickle cell disease who were receiving hydroxyurea for periods of 5-15 monthson four consecutive days with escalating dose of recombinant erythropoietin (EPO) for 7 weeks, given on the alternate three days along with oral iron sulfate. Treatment with EPO in combination with chronic hydroxyurea therapy had a significant effect on the percentage of hemoglobin F containing reticulocytes (F-reticulocytes), on red cells (F-cells) and on the total hemoglobin F level. In these four patients such combination therapy was associated with a 1.4 to 3 fold increase in F-reticulocytes, a 1.3 to 2 fold increase in F-cells and a 1.4 to 2 fold increase in the percentage of hemoglobin F when compared to the maximal values previously achieved on hydroxyurea alone. In addition, in contrast to the results on hydroxyurea alone, treatment with combination HU/EPO resulted in maximal stimulatory effects within ten to twelve days of therapy. This additional augmentation resulted in a further decrease in serum bilirubin and lactate dehydrogenase as well as a further decline in intracellular hemoglobin S polymerization tendency at physiologic oxygen saturation in cells containing fetal hemoglobin. In three patients in whom it was measured, there was a concomitant improvement in red-cell filterability as measured with a nickel mesh network of uniform 3 micron pore size. Changes in filterability closely parallel hydroxyurea/recombinant erythropoietin induce changes in fetal hemoglobin level. We conclude that recombinant erythropoietin when given in an alternating fashion with erythropoietin in addition to iron supplementation tends to augment the fetal hemoglobin responses seen with hydroxyurea alone. This observation indicates that Epo may offer a hydroxyurea sparing effect which would be of particular importance in individuals who are likely to require hydroxyurea therapy for extended periods of time.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25064-05 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cytogenetic Investigations of Patients with Genetically Determined Disorders

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Beverly J. White	Director, Cytogenetic Unit	LCB, NIDDK
Others:	Damrong Wangsa	Photographer, Scientific Technical	OD, CC
	Flanagan Whitsitt	Medical Technician	OD, CC
	Cynthia Powell	Clinical Associate	OD, CC

COOPERATING UNITS *(if any)*
Medical Genetics Program, CC (W. Gahl, D. Parry); DE, NICHD (G. Merriam, L. Nelson); LN, NIA (M. Schapiro, S. Rapoport); LCS, NIAAA (M. Filling-Katz, D. Goldman); Cytogenetics Laboratory, Children's Hospital, Washington, D.C.. (W. Stanley)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Molecular Forces and Assembly (Cytogenetics Unit)

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 4	PROFESSIONAL: 3.0	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cytogenetic studies of 215 new clinical center patients were completed and correlated with diagnostic information. Abnormal karyotypes were frequent in infertility or hypogonadism (28.1%) and idiopathic short stature (11.5%), and increased in premature ovarian failure (3.2%). Abnormalities in short stature usually involved the sex chromosomes (70%). Of 77 Turner syndrome patients, 71.5% were nonmosaic 45, X, and 28.5% had 45, X mosaicism or a structurally abnormal X. All 19 new patients with mental disorders were fragile-X negative; one carried a familial autosomal rearrangement which will be further investigated.

Our ovarian failure results were submitted for publication, together with data from a clinical trial of gonadotropin suppression treatment. Findings in familial parathyroid carcinoma cell cultures were also submitted for publication; clones with an autosomal translocation, chromosome 7 aneuploidy, and chromosome 9 inversion were identified in the tumor. CNS studies of young adult fragile X patients are being reported and include our determination of fragile-X expression levels. Completed studies of mosaic trisomy 21 in a non-mentally retarded adult with presenile dementia and a family with carotid body tumor with pheochromocytoma were published.

We plan to continue these patients investigations. Our goal will be to correlate cytogenetic findings with molecular analyses and clinical status. Variations of the sex chromosomes will be emphasized.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-938

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25066-05 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
AIDS: Transcriptional Regulation by TAT-Protein and LTR of HIV In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jiangang Yuan	Visiting Associate	LCB, NIDDK
Others:	Tiee-cherng Shieh	Visiting Fellow	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
Kabigen, Stockholm, Sweden (Professor M. Hartmanis)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 1.2	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human immunodeficiency virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS). Expression of this virus is regulated by several proteins, one of these proteins is TAT which has the ability to autoregulate the expression of HIV by promoting the transcriptional activity of HIV-LTR. The region of HIV-LTR needed for TAT mediated regulation was termed TAR, and the sequence between nucleotide plus 14 to plus 45 relative to the transcription initiation site was shown to be essential for the response to TAT protein.

We have previously shown by using in vitro transcription assays that the purified recombinant TAT protein increased the trans-acting activity significantly, but the enhancement of purified TAT protein did not reach the levels obtained with nuclear extract from HeLa cells transformed with TAT gene. The results of gel retardation assay showed an extra band exhibited with HeLa/c TAT nuclear extract. These data suggest the possibility of another important nuclear factor which is only present in HeLa cells transfected by TAT gene. In order to provide more evidence for this cellular nuclear factor's function, we have scaled the HeLa/c TAT cell production from 3 ml to 40 ml pellets of cells and developed a gradient Heparin-Sepharose chromatographic method for fractionation of the HeLa/c TAT nuclear extract. After fractionation we found that different fractions had different gel retardation patterns. The results of in vitro transcription assay showed that only the fractions, which contained the specific binding cellular factor, could significantly increase the trans-acting activity from HIV-LTR promoter. The TAR DNA sequences acted as a strong competitor for the enhancement activity of this factor. In vitro, 40 fold molar excess of TAR competitor relative to the amount of DNA template was able to reduce the trans-activation of the HIV-LTR to the basal level. These results suggest that a specific factor in HeLa/c TAT cells is involved in the TAT protein's function and the effect may be mediated at the DNA level. The presence of a specific cellular factor in HeLa/c TAT cells could provide a new insight into the mechanism of TAT protein's function.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25069-03 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Coordinated Expression of Human Beta Sickle Antilles and Human Alpha Globin in Transgenic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Frank Shafer	NRSA Fellow	LCB, NIDDK
Others:	Zi Yao Liu	Visiting Associate	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
DMBN, NIND (Drs. S. Karlsson and B. Dropulic); University of South Carolina, Columbia, S.C. (Dr. M. Dewey); Nippon University, Tokyo (Dr. N. Uyesaka)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Project Z01 DK 25016-18 LCB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25070-03 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of the Epsilon Globin Gene Flanking Sequences

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Natalya Merezhinskaya	Visiting Fellow	LCB, NIDDK
Others:	Panagoula Kollia	Visiting Fellow	LCB, NIDDK
	Kyung Chin	Research Biologist	LCB, NIDDK
	Constance Tom Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
Hahneman Medical School (Dr. Doris Morgan), Philadelphia, Pennsylvania

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.0 PROFESSIONAL: 1.0 OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The K562 erythroleukemia cell line constitutively expresses low levels of embryonic and fetal but not adult hemoglobin and can be further induced by chemical stimuli for hemoglobin production. Within the cell, regulation of transcriptional activity of globin genes is determined by a variety of cis-acting regulatory DNA sequences and trans-acting proteins or other factors. Several cis-acting sequences have been identified through naturally occurring and laboratory synthesized mutations or deletions. One of these is the silencer located in the region between about -250 and -300 5' in the epsilon-globin gene which has been identified by deletion mutations in transient transfection assays.

To examine in detail the molecular mechanism of the epsilon-globin gene silencer activity and its possible role in the developmental regulation of epsilon-globin gene expression, further characterization of the epsilon-globin gene silencer is necessary. In particular, determination of the silencer activity in non-deleted or non-truncated mutations of the epsilon-globin gene is essential. We have mutated six nucleotides in the silencer region of an epsilon-globin gene containing 1.4 kb of 5' flanking sequence. The activity of this 1.4 kb 5' flanking sequence fused to a reporter gene (luciferase) was 3 to 6 fold greater than the wild type sequence when assayed for transient expression in K562 cells. This study confirms the importance of the epsilon-globin gene silencer and supports its possible role in regulation of developmental expression. The deletion and site directed mutations of the epsilon-globin gene will be examined for their ability to correctly initiate transcription as a possible mechanism of action for the silencer.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25071-03 LCB

PERIOD COVERED

October 1, 1990 to September 31, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-Regulation of Human Globin Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Robert H. Broyles	Guest Worker	LCB, NIDDK
Others:	Patricia Berg	Expert	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Department of Biochemistry, University of Oklahoma School of Medicine (Dr. R. Broyles)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.2 PROFESSIONAL: 0.2 OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Understanding the molecular regulation of developmental hemoglobin switching would be useful for increasing fetal hemoglobin in adult humans with sickle cell anemia and other hemoglobinopathies, a manipulation likely to alleviate the clinical manifestations of the disease. The goal of this project is the identification and isolation of trans-acting factors that regulate hemoglobin switching, particularly protein regulatory factors that bind to the 5' noncoding regions of the human beta globin gene.

Hypothesis: The observation that K562 cells, and embryonic erythroid cells, have a large amount of ferritin but do not express adult beta globin, whereas adult erythroid cells have opposite characteristics, led us to hypothesize a link between the regulation of iron metabolism and regulation of globin genes - particularly that a subunit of ferritin (or a structurally-related protein) is a repressor of adult beta globin expression in K562 cells and mediates its effect by binding to the DNA and/or associated proteins of one or more of the 5' control regions of the beta globin gene.

Results: H and L ferritins and a partially purified ferritin-like protein from K562 cell nuclear extracts bind to the 5' beta Rsa fragment (-223 to -129) whereas transferrin and apotransferrin do not. The binding of the K562 ferritin preparations increases with the amount of protein added. Gel shift experiments with anti-ferritin antisera show that ferritin subunits or a ferritin-like protein(s) present in all K562 nuclear extracts is responsible for this binding. Gel shifts with nuclear extracts, anti-ferritin antiserum, and oligonucleotides have narrowed the binding site to a 37bp stretch (-165/-129) within a putative negative regulatory region. Interestingly, this region contains a DNA version of the consensus hexanucleotide (CAGTGN) of an iron responsive element (IRE). Competition gel shifts with mutated oligonucleotides and DNase-I footprinting are in progress to further localize the binding site, and the binding protein is also being further purified.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK-25073-03 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Erythropoietin Receptor and Its Genetic Control in Red Cell Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donna M. Williams	Special Volunteer	LCB, NIDDK
Others:	Kyung Bae	Guest Worker	LCB, NIDDK
	Teresa A. Zimmers	Special Volunteer	LCB, NIDDK
	Kyung Chin	Biologist	LCB, NIDDK
	Yuko Wada	Visitng Associate	LCB, NIDDK
	Griffin P. Rodgers	Chief, Molecular Hematology	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
Johns Hopkins University, Baltimore, MD (W. David Hankins); George Mason University, Fairfax, VA (Feng-Sheng Xu and W. David Hankins); Massachusetts Institute of Technology, Cambridge, MA (Gregory Longmore)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 3.0 PROFESSIONAL: 3.0 OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To study the mechanism by which the kidney hormone, erythropoietin, regulates normal and abnormal erythropoiesis, we have isolated and sequenced the genomic and cDNA genes for the human erythropoietin receptor (ER). The transcription initiation site was determined to be at approximately -134 bases upstream of the first codon. Based on the sequence information obtained from the genomic clone, primers were designed which allowed the construction by polymerase chain reaction (PCR) techniques of a human ER cDNA clone containing from -10 bases upstream of the translation start site to 27 bases past the translation stop site. With the possession of the cDNA clone which allowed the construction of a competitive DNA template, we were able to develop a PCR assay for the quantitation of the human ER in both tissue culture cells and peripheral blood samples. The number of ER transcripts per nucleated cell from the peripheral blood of two normal individuals was determined to be on the order of magnitude of 10^{-3} copies per cell. We believe that expression of the ER gene is indicative of an erythroid progenitor cell and that the small number of ER transcripts observed in these samples is consistent with the expectation that only a very minor population of erythropoietin responsive progenitor cells exists in the peripheral blood of normal individuals. The cloning and sequencing of the genomic and cDNA ER genes has allowed the development of a method for quantitation of low levels of ER mRNA from small amounts of starting material. This approach is being tested as a new method for detection and monitoring of erythroid progenitors in patients with sickle cell anemia who are under erythropoietin and hydroxyurea therapy. In addition, transcriptional regulatory sequences and receptor function are being studied by deletional and site directed mutagenesis.

U.S. GOVERNMENT PRINTING OFFICE: 1991

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25074-03 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism(s) of Enhanced Gamma Globin Gene Expression in Patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	In-Hoo Kim	Visiting Associate	LCB, NIDDK
Others:	Griffin P. Rodgers	Chief, Molecular Hematology Unit	LCB, NIDDK
	Pat Berg	Expert	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.8
PROFESSIONAL: 0.8
OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Several lines of clinical and experimental evidence suggest that elevated levels of fetal hemoglobin (HbF) may improve the clinical course of individuals with sickle cell disease and beta thalassemia. A number of cytotoxic drugs have been shown to enhance gamma globin synthesis (and HbF levels) in experimental animals and patients with hemoglobinopathies, although the mechanisms of action of these agents have yet to be clearly delineated. The K562 human erythroleukemia cell line has been shown to express constitutively low levels of embryonic and fetal but not adult hemoglobin, and can be reversibly induced to increase preferentially gamma-globin gene expression in response to hydroxyurea. We are, therefore, using the K562 cell as a model system to understand the mechanism by which hydroxyurea (and similar agents) induces fetal hemoglobin synthesis. K562 cells have been grown in the presence of 25mM hemin and 100mM hydroxyurea. Nuclear extracts have been prepared from these cells. A 420 bp fragment 5' of the normal A-gamma gene has been subcloned into an expression vector, and has been used to examine the differential binding characteristics of the nuclear protein extracts. We have employed gel-retardation electrophoresis, DNA-footprinting (DNaseI and methylation protection), in vivo genomic DNA footprinting, and ion exchange and affinity chromatography to investigate the interaction of both specific and non-specific protein interaction with the treated A-gamma gene promoter DNA. By gel shift analysis, we find a strong band in the hemin treated cells. This band cannot be competed for by OCTA-1, NF-1, and GATA-1. This suggests the possibility of novel binding activity underlying the known hemin effect. We will localize and specify this binding site by DNaseI footprinting. In hydroxyurea treated cells, however, we see an inconsistent shift band by gel shift assay. Current studies have been developed employing in vivo genomic DNA footprinting analysis to determine the site and specificity of the hemin effect and the putative hydroxyurea effect. It is hoped that the further identification, characterization and purification of this protein would not only extend the current knowledge of the molecular basis of the fetal to adult "switch", but also suggest novel pharmacological approaches to the reversal of this switch in several clinically significant hemoglobinopathies.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-938

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25075-03 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Production and Characterization of HIV TAT

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Tiee-Cherng Shieh	Visiting Fellow	LCB, NIDDK
Others:	Jian-gang Yuan	Visiting Fellow	LCB, NIDDK
	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
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LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Project ZO1 DK 25066-05 LCB.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-838

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25076-02 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of Transcription Factors Binding to the Epsilon Globin Silencer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Betty Peters	Robert Wood Johnson Fellow	LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Yuko Wada	Visiting Associate	LCB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.2
PROFESSIONAL: 1.2
OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hemoglobin switching is an example of tissue and temporal regulation of gene expression. Embryonic globin chains (zeta and epsilon) are expressed during the first trimester of gestation. Fetal globin genes (alpha and gamma) are the predominant globin chains expressed during the second and third trimester of birth. After birth alpha and beta globin are the predominant globin chains expressed. There is evidence that competition for trans-acting factors is important for appropriate developmental regulation of gamma and beta globin gene expression; however, epsilon globin gene expression exhibits appropriate developmental regulation even in the absence of other beta-like globin genes (gamma and beta) indicating that competition for factors with the beta and gamma gene is not required for appropriate developmental regulation of epsilon globin. The epsilon globin gene and flanking DNA and trans-acting factors are important for autonomous regulation of epsilon gene expression. The epsilon globin silencer has been shown to be important for autonomous regulation of epsilon. Previously two factors have been shown to bind to the silencer, an erythroid specific and a ubiquitous factor. We have begun characterizing the activities of these factors. We have evidence that one factor is a transcription activator and that the other is a transcription inhibitor. Using mobility shift assays we have also shown that these factors bind to overlapping regions and likely compete for binding to the silencer. We are in the process of purifying these factors using protein chemistry and molecular genetic approaches.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-930

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25077-02 LCB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Beta-Globin Gene Expression in Patients with Different Types of Beta-Thalassemia Mutation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Shu-zhen Huang, M.D.	Visiting Associate	LCB, NIDDK
Others:	Griffin P. Rodgers	Chief, Molecular Hematology	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS *(if any)*
Institute of Medical Genetics and the Children's Hospital, Shanghai, China (Dr. Y.-T Zeng)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.4 PROFESSIONAL: 1.4 OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The beta-thalassemias are a group of inherited blood disorders characterized by decreased (beta-plus) or absent (beta-zero) beta-globin synthesis. Recent studies have revealed that the disease results from a heterogeneity of point mutations on the beta-globin gene locus. However, little is known about the mRNA expression for each of the mutant beta-globin genes. Recently, mutations at IVS-2 region of the beta-globin gene have been of general interest as it is evident that this intervening sequence plays an important role in regulation and expression of the gene. Beta-IVS-2 nt. 654 cytosine-to-thymine is a common Chinese beta-thalassemia mutation. In order to investigate the mRNA expression and the splicing defect of this specific mutation, we used our newly developed method, i.e., enzyme reverse transcription and polymerase chain reaction (RT-PCR) to amplify cDNA copies of reticulocyte mRNA followed by direct sequencing of the amplified cDNA, to analyze the mutant mRNA transcript. The result showed that, in the homozygous patient, two principle unusually long amplified fragments of 254-bp and about 290-bp were observed. Direct sequencing of the patient's amplified cDNA revealed that the 181 -bp fragment had a normal sequence, but in the 254-bp fragment, the new donor-like site due to the IVS-2 nt. 654 cytosine-to-thymine substitution, was exactly spliced to the normal IVS-2 3' acceptor site and an internal cryptic acceptor site at position of IVS-2 nt. 579 was exactly spliced to the normal IVS-2 5' donor site. These and other data indicate that IVS-2 nt. 654 cytosine-to-thymine mutant gene does not abolish the normal beta-globin mRNA processing entirely but does produce a small amount of normal spliced beta-globin mRNA. Hence IVS-2 nt. 654 mutation should be classified as beta-plus thalassemia at mRNA level. Current studies in progress are aimed at: (1) quantitation in absolute term of globin mRNA amount in erythroid cells from the patients with IVS-2 nt. 654 cytosine-to-thymine mutation as well as other known beta-thalassemia mutations using PCR techniques; (2) investigation of the expression of IVS-2 nt. 654 and other known mutant genes at translational level using globin chain biosynthesis with HPLC techniques. Thus, the overall objective is to elucidate the relationship between specific mutations and beta-globin gene expression. On this basis, we will be able to contribute to the knowledge of the genetic heterogeneity of beta-thalassemia as well as provide new information on gene structure and function in eukaryotic cells.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-654-[illegible]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK-25078-01

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Globin Expression in an Erythroid Progenitor Culture System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lillian Burke	NRSA Fellow	LCB, NIDDK
Others:	Eitan Fibach	Special Volunteer	LCB, NIDDK
	Donna Williams	Special Volunteer	LCB, NIDDK
	Panagoula Kollia	Visiting Fellow	LCB, NIDDK

COOPERATING UNITS *(if any)*
Transfusion Medicine, CC (M. Fox); Clinical Pathology, CC (T. Fleischer); Walter Reed Army Medical Center (D. Wright)

LAB/BRANCH	Laboratory of Chemical Biology
SECTION	Section on Molecular Biology and Genetics
INSTITUTE AND LOCATION	NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.6	PROFESSIONAL: 1.6	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The gamma to beta switch in the synthesis of hemoglobins is not well understood. The primary model system for studying this process, the K562 cell line, does not synthesize the beta globin. Recently, the conditions for culture of erythroid progenitors from peripheral blood have been described. Preliminary studies using RNAse protection assays and RNA hybridization indicate that these cells reproduce the gamma to beta switch in culture.

An erythroid progenitor culture system has been set up and we are establishing the culture conditions necessary to obtain consistent globin synthesis. Protocols have been established to extract RNA and perform the RNAse protection assay to quantitate the specific globin mRNAs produced. A variety of cell staining techniques have been investigated; the May-Grunwald-Giemsa and benzidine stains are presently in use.

We plan to use the erythroid progenitor culture system to study the transcriptional control of switching during erythroid maturation, during the early post-natal period (using cord blood), and in switching mutants such as Hereditary Persistence of Fetal Hemoglobin and certain forms of beta thalassemia. Other planned studies include: studies of the mechanism of transcriptional alteration of globin expression by agents such as hydroxyurea, oxygen, erythropoietin, and iron; studies of the development and terminal differentiation of erythroid progenitor cells; and, ultimately, studies of the proteins involved in stage-specific transcriptional control.

This system allows us to reproduce, in culture, the normal terminal differentiation of the erythroid progenitor cell and to correlate this maturation with the production of specific globin mRNA. It will allow the development of studies which are important both clinically and in several areas of basic medical research including: the molecular biology of normal development and maturation, the isolation and study of the proteins involved in the regulation of globin expression, the pharmacology of drugs and chemicals which affect globin transcription and which might be useful in the therapy of sickle cell anemia and beta thalassemia, and studies of the erythropoietin receptor, among others.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-438

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research in the Laboratory of Chemical Physics is primarily concerned with problems in structural biology and biophysics. There are direct applications of much of this work to the pathophysiology and treatment of human diseases such as AIDS, malaria, and sickle cell anemia. A variety of structural and spectroscopic techniques are employed in these investigations, including nuclear magnetic resonance (NMR), Raman and infrared spectroscopies, time-resolved optical spectroscopy with nanosecond and picosecond lasers, and non-linear optical spectroscopy. There is also a major effort in theoretical studies to complement the experimental work, including both analytical methods and the use of high speed computers in large scale calculations. The systems under study include small prototypical molecules, nucleic acids, proteins, intact and model membranes, retinal photoreceptors, and flagellar motors. Current research focusses on : the development and application of new NMR methods to the determination of the three-dimensional structure of polypeptides and proteins in solution, conformational and dynamical properties of membrane systems, dynamics of ligand binding and conformational changes in proteins, theoretical analysis of kinetics and chemical dynamics, computer simulations of protein motions, the analysis of excited electronic states of polyenes, the molecular mechanism of excitation in photoreceptor cells and ionic processes in cell membranes, the gelation of hemoglobin S and its relation to the pathophysiology and therapy of sickle cell disease, and the synthesis of potential therapeutic agents for malaria.

Bax and colleagues continue to make major advances in the development of multi-dimensional NMR methods for the determination of the three dimensional structure of increasingly larger proteins in solution. The new methods are less susceptible to problems created by large resonance linewidths, which are approximately proportional to the molecular weight of the protein. One example is a four-dimensional NMR experiment (in collaboration with Clore and Gronenborn) which dramatically reduces spectral overlap in one of the most crowded regions of the NOE spectrum, thereby permitting the study of the interaction between aliphatic residues in proteins of substantial size. The new experiments provide access to a large number of parameters that contain structurally important information. In another study, comparison of ^{13}C chemical shift data with protein structures indicates that there is a clear correlation between chemical shifts and backbone torsion angles that is expected to of major use in obtaining resonance assignments in proteins. The new techniques developed by Bax and colleagues have been used to study the structure of a number of proteins, the complex of calmodulin and a fragment of myosin light chain kinase.

Clore, Gronenborn and colleagues have developed and exploited multi-dimensional NMR techniques to investigate the three-dimensional structures of a large number of proteins in solution. In this work they have demonstrated that it is possible to determine the structure of proteins in solution by NMR with an accuracy comparable to that of high resolution X-ray crystallography. The structures studied during the past year include the ribonuclease H domain of HIV-1 reverse transcriptase, the p7 nucleocapsid protein of HIV-1, interleukin 1ß, and domains from thioredoxin, the human enhancer binding protein, and protein G of Streptococcus. 3D double and triple resonance NMR spectroscopy has been used to obtain ^{1}H, ^{15}N, and ^{13}C backbone sequential assignments for the ribonuclease H domain. The observed secondary structure in solution is consistent with that found in the crystal structure, but there appears to be substantially more disorder at the C-terminus in the solution structure. A comparison of the two zinc finger domains of the p7 nucleocapsid protein of HIV-1 shows that the polypeptide backbones have identical conformations. 3146 NMR constraints have been used to determine a very accurate structure for the 153 residue protein, interleukin 1ß. Even though this is the largest protein structure solved so far by NMR, the large number of NMR constraints permitted the determination of the atomic coordinates of the backbone atoms to 0.4 A and all atoms to 0.8 A. Comparison of the structure with an analysis of functional studies by others on mutants, points to three distinct binding sites on the protein surface for the receptor. Clore and Gronenborn have also determined a very accurate structure for the 55 residue IgG binding domain of protein G, a cell surface protein of the bacterium Streptococcus. This protein is extremely stable, which may be explained by a combination of the large number of hydrogen bonds involved in ß sheet formation, its distribution of hydrophobic and hydrophilic residues, and its novel polypeptide fold.

Becker and colleagues have been developing new NMR methods to study the structure of small molecules oriented in liquid crystals. They are also exploring the mechanism and potential applications of the effect of isotopic substitution on NMR chemical shifts of nuclei up to 10 bonds away from the site of substitution.

Szabo and coworkers have carried out several theoretical investigations of dynamical processes. Analysis of the geminate rebinding of carbon monoxide to protoheme shows that the non-exponential time course can be accurately predicted, not just fitted, using a theory for diffusion-influenced reactions. In another study, the validity of Smoluchowski theory for irreversible reactions has been demonstrated by comparing the theoretical predictions with results of molecular dynamics simulations of fluorescence quenching in a hard sphere liquid as "experimental data". The influence of diffusion on chemical reactions has also been investigated for the case of reversible, bimolecular binding, yielding results in contrast to predictions of ordinary chemical kinetics. Along similar lines, a novel and efficient method using Brownian dynamics has been developed for calculating the time-dependent rate coefficient of a bimolecular diffusion-influenced reaction between species with complex interactions.

During the past year the focus of Zwanzig's research has been on studies of diffusion influenced reactions. One investigation (in collaboration with Szabo) led to a new time-dependent version of Berg and Purcell's steady state theory of diffusion-influenced ligand binding to receptors randomly distributed on the surface of a cell. In another, theory and computer simulations were applied to a model for a chemical reaction with a rate controlled by an entropy barrier (e.g. a steric bottleneck to the motion of a ligand through a protein), rather than the more common potential energy barrier. In a third investigation, considerable progress has been made in developing a general theory for reversible, diffusion-influenced bimolecular reactions.

Chen has carried out several theoretical studies on bioenergetics and kinetics. In one, the dequenching of fluorescent probes used to measure the kinetics of cell fusion has been modelled by solving time-dependent diffusion equations. In another, a Monte Carlo simulation is being used to study the dynamics of the rotation of a flagellar motor. Also, a general formalism has been obtained for the calculation of binding isotherms for binding two or more long ligands to a one-dimensional lattice to which more than one ligand can bind simultaneously, and applied to a case of protein-protein binding.

Rubin has obtained an explicit analytic expression for the probability that a random walk will be trapped at a particular trapping site in a one-dimensional random walk with equally spaced traps on the lattice.

McDiarmid is carrying out optical spectroscopic studies on the electronic structure and conformation of the excited states of small polyatomic molecules that are models for more complex biological molecules. A study of the absorption spectrum of acetone in the vacuum ultraviolet has led to a new model for its photodissociation.

Levin, Lewis and coworkers are using Raman and infrared spectroscopy to investigate the structural and dynamical properties of both model and intact biological membranes. Studies on the gel to liquid crystalline phase transitions of reconstituted membranes indicate that the interaction of the lipid head groups with the charged amino acid side chains of peptides derived from human surfactant protein SP-B are important features of a functionally successful lung surfactant. Other packing properties of lipid bilayers are also being studied, including an investigation of the interdigitated membrane state from a systematic study of the phase diagram for a binary mixture of a symmetric and highly asymmetric lipid. A novel spectroscopic imaging technique is being developed which combines the spatial resolving power of optical microscopy with the selectivity of vibrational spectroscopy. Another new technique being developed is the use of resonance Raman spectroscopy to monitor the redox, spin, and oxidation state of the membrane protein, cytochrome oxidase, as a function of a potentiometrically-controlled solution potential.

Eaton, Hofrichter and Henry are using time resolved optical spectroscopy in photodissociation experiments to investigate structure function relations in myoglobin and hemoglobin. The technique of high-precision nanosecond spectroscopy in partial photolysis experiments has been developed and has led to several important new findings, including (i) demonstration that the transition state for the quaternary structural change of hemoglobin is much more like the R conformation than the T conformation, a result which explains the linear free energy relation between the quaternary rates and equilibrium constants, (ii) discovery of the protein relaxation subsequent to photodissociation of the myoglobin carbon monoxide complex that may be responsible for a time-dependent barrier to ligand rebinding, (iii) and demonstration in hemoglobin that there is no communication between subunits prior to the quaternary conformational change at about 10 microseconds. In all of these experiments singular value decomposition has played a key role in the data processing and analysis. Henry and Hofrichter have used both analytic methods and computer simulations to study the application of singular value decomposition to the analysis of experimental data.

Studies by Hofrichter and Eaton on the domain structure and kinetics of hemoglobin S gel formation support the hypothesis that homogeneous nucleation of a single polymer molecule can trigger the formation of an entire domain of polymers, and therefore that variations in the rate of homogeneous nucleation can account for the wide variety of shapes for deformed cells in sickle cell anemia.

Hofrichter and Clore have carried out a systematic analysis of kinetic data on the photocycle of bacteriorhodopsin. Detailed modelling has shown that back reactions are significant for all steps of the cycle except the last.

Hagins and Yoshikami are investigating the mechanism of visual transduction. Improvements and extensions of pyroelectric calorimetry for study of heat production by milligram weight biochemical and cellular systems are under development. Pyroelectric calorimetric studies of phototransduction in frog retinas are being extended to low light exposures and to retinas that have been subjected to sudden changes in their fluid environment. A series of new compounds have been synthesized to be useful as elemental standards for estimating water content and transmembrane potentials in retinal and brain cells. Distributions of these compounds is being studied by electron energy-loss spectrometry. The metabolism of esterified membrane-permeable fluorescent calcium indicator dyes is being studied by high-pressure liquid chromatography and mass spectrometry, with the aim of explaining the uneven cellular distribution of the dyes and the presence of fluorescent but calcium-insensitive forms in retinal photoreceptors.

Ziffer is synthesizing novel compounds that are potential agents for the treatment of malaria. The fungus *Beauveria sulfurescens* has been used to introduce hydroxyl groups on unactivated methyl, methylene and methine groups of artemisinin derivatives. When arteether, the ethyl ether of dihydroartemisinin, was employed as the substrate, two hydroxylated derivatives were isolated - 14-hydroxyarteether and 9ß-hydroxyarteether. These derivatives are approximately as active as arteether, and work is in progress to prepare larger quantities for testing in mice.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29001-19-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Molecular dynamics and vibrational characteristics of membrane assemblies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.:	Ira W. Levin	Research Chemist	LCP-NIDDK
Others:	E. Neil Lewis	Visiting Associate	LCP-NIDDK
	James L. Slater	IRTA	LCP-NIDDK
	Paul Harmon	IRTA	LCP-NIDDK
	Patrick Treado	IRTA	LCP-NIDDK

COOPERATING UNITS *(if any)* R. Adams, LCP-NIDDK; C. Huang, School of Medicine, Univ. of VA; J. S. Vincent, Univ. of MD.; R. Hendler, NHLBI; B. J. Litman, School of Medicine, Univ. of VA; C. G. Cochrane, Research Institute of Scripps Clinic, CA.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
4.5	4.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Vibrational Raman and infrared spectroscopy are used to probe the dynamical, conformational, functional and thermodynamic properties of both model and intact membrane assemblies. Emphasis is placed on elucidating lipid-lipid and lipid-protein interactions within the bilayer aggregate. For example, model bilayer systems were reconstituted with several synthetic peptide paradigms for modeling human lung surfactant. The synthetic peptides consisted of residues 59-81 of human surfactant protein SP-B and twenty-one amino acid residue peptides containing repeating units of arginine separated by either four or eight leucines. Raman spectroscopic data demonstrated that the membrane bilayer order induced by constraints resulting from the interaction of charged amino acid residues with acidic bilayer lipids are critical features of a functionally successful lung surfactant.

A novel approach has been developed for characterizing the redox, spin and conformational states of cytochrome oxidase by simultaneously determining the resonance Raman vibrational spectra and optical absorption spectra as a function of a potentiometrically controlled solution potential. As an adjunct, the first potentiometric titration of ferricytochrome c monitored by resonance Raman spectroscopy was determined.

We are developing a novel spectroscopic imaging technique which combines the spatial resolving power of optical spectroscopy with the sensitivity and selectivity of vibrational spectroscopy. Using a commercial optical microscope, state-of-the-art acousto-optic devices and an extremely low light level imaging detector, our instrument should be capable of diffraction limited spatial resolution for both Raman scattering and near-infrared absorption spectroscopic microscopy.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-29005-17

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
Asymmetric synthesis; structure, stereochemistry, and NMR

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P. I. :	Herman Ziffer	Research Chemist	LCP-NIDDK
Others:	Yuming Pu	Visiting Fellow	LCP-NIDDK
	Yulin Hu	Guest Worker	LCP-NIDDK

COOPERATING UNITS *(if any)*
Dr. Sanford Markey, NIMH, LCS; Dr. R. J. Highet, NHLBI, IR CH; Dr. Raymond Chen, NHLBI, IR, LC.

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

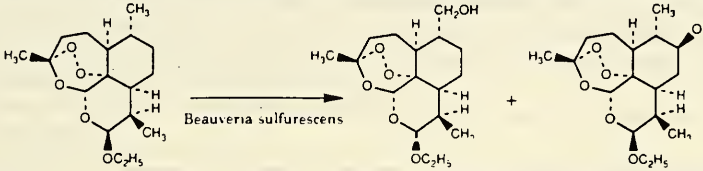
INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

The formidable problems encountered in developing a malaria vaccine, and the ability of *Plasmodium falciparum* to develop resistance to new drugs, have stimulated interest in using combinations of drugs for treating this disease, which claims more than a million lives a year. Although strains of *P. falciparum* have become resistant to many drugs, no strains have thus far become resistant to derivatives of artemisinin. Artemisinin derivatives are thought to damage the parasite's membranes, while quinine or mefloquine inhibit dihydrofolate reductase. Thus, the parasite has to mutate at a different locus to become resistant to artemisinin. A large number of derivatives (esters and ethers of dihydroartemisinin) have been prepared in China and the United States and shown to be active against *P. falciparum*. For additional structure-activity studies, we wanted to introduce a new functional group into the molecule, without destroying the peroxide necessary for its antimalarial activity. We have investigated the ability of the fungus *Beauveria sulfurescens* to introduce hydroxy groups on unactivated methyl, methylene and methine groups in artemisinin derivatives. In an earlier study we found that *B. sulfurescens* introduced a hydroxy in low yield on the C-14 methyl of the N-phenylurethan of the dihydroartemisinin. When arteether, the ethyl ether of dihydroartemisinin, was employed as a substrate with the same fungus two hydroxylated derivatives were isolated, 14-hydroxyarteether and 9β-hydroxyarteether. The yields were greater than that obtained from the N-phenylurethane of dihydroartemisinin. *In vitro* studies of several derivatives prepared to date show them to be approximately as active as arteether. Work is in progress on scaling up these reactions, so as to prepare larger quantities of the compounds for testing in mice.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29006-21-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

The structure and dynamics properties of macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I. :	Elliot Charney	Scientist Emeritus	LCP-NIDDK

COOPERATING UNITS (if any)

H-H Chen, George Mason University, Fairfax, VA; Rodney Harrington, University of Nevada, Reno, NV; D. C. Rau, LCB-NIDDK

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Laboratory of Chemical Physics

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Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

Macromolecular structure, dynamics and polyelectrolyte properties of large biological polymers, in particular, polynucleotides and nucleic acids are being studied by electric-field induced dichroism and birefringence methods.

The current research is a response to the fact that the knowledge of the structural effects of specific base-pair sequences on DNA translation and replication is still at a primitive stage. Only one or two biologically significant protein-DNA complexes from which such structural effects could be inferred have been crystallized and their structure determined. Using electro-optic birefringence and dichroism, it is now possible to quantitatively explore DNA structures in solution, albeit with less resolution than x-ray diffraction of crystals, but uninhibited by the problem of forming crystalline complexes. The principal project currently being pursued is the structural effect of the triplet sequence CAC/GTG.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29007-20-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and interaction of biomolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. :	Hideo Kon	Research Chemist	LCP-NIDDK

COOPERATING UNITS (if any)

Sumihare Noji, Okayama University, Okayama, Japan

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INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

During the period of October 1, 1990 - March 31, 1991, the particular phase of the project that concerns Deformability of Red Blood Cells using Electron Paramagnetic Resonance (EPR) was reviewed. This phase of the general project, Structure and Interaction of Biomolecules, started in 1980 with a novel idea of using spin labeling technique for probing the slow dynamics of macroscopic (e.g. cellular) state, while the spin labeling technique had been used exclusively to study the molecular (microscopic) motional state and its change. Our idea was based on the change of EPR spectral shape derived by partially orienting the cells in shear flow, the ease of orientation being directly related to the ease of cell deformation by flow. First, a theoretical model with the cell deformability included as a parameter was constructed to explain and simulate the observed EPR spectra, enabling to estimate the degree of orientation and deformation index from spectral changes in flow. Decay in time of the flow-induced spectral change was also used to judge the cell deformability. The method was applied to study red blood cells in the states where the cell deformability was altered for various reasons: for example, the effects on the cell deformability of spin labeling itself, cross-linking by diamide, glutaraldehyde and other chemicals, effect of intracellular Ca^{2+}, Cu^{2+} ions, Heinz body formation, hematocrit, effect of co-existing hardened cells. Red cell ghosts were also studied with a specific interest in finding among various modifications of ghost preparation method the one that gives the best deformation properties, and finding a possible relation between an altered state of cytoskeletal protein spectrin and flow properties of ghost. in most cases, the factor(s) were identified in the cell structure that cause the deformability change.

The project 29007 is terminated as of March 31, 1991 due to retirement of the principal investigator.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29008-20-LCP

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Electric and molecular structural investigation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Ruth McDiarmid	Research Chemist	LCP-NIDDK
Others:	Aharon Gedanken	Special Volunteer	LCP-NIDDK

COOPERATING UNITS (if any)
Leo Klasinc and Branka Kovać, Rudger Bošković Institute, Zagreb, Yugoslavia; Robert Wu, Univ. Southern California, Los Angeles, CA

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NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The one- and two-photo resonant spectra of the 193 nm region of the spectrum of cyclopentadiene was measured and analyzed. The optical (1-photon) spectrum was shown to be composed of two interleaved 3p-Rydberg <- X transitions. From these spectra, the C=C bonds were deduced to be elongated in these excited states. The 2-photon spectrum was shown, by polarization-selection, to be dominated by totally symmetric false origins to two of the 3p-Rydberg <- X transitions.

The absorption spectrum of the 3s-Rydberg <- X transition of acetone was measured and analyzed. The previously proposed analysis of this spectrum and model for 193 nm photodissociation of acetone were demonstrated to be incorrect.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29010-19-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Dynamics of Proteins and Studies on Sickle Cell Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	William A. Eaton	Medical Officer	LCP-NIDDK
Others:	James Hofrichter	Research Chemist	LCP-NIDDK
	Eric R. Henry	Research Physicist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen M. Jones	I.R.T.A.	LCP-NIDDK
	Garrott R. Christoph	Expert	LCP-NIDDK

COOPERATING UNITS *(if any)*

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, Italy

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Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Time resolved optical spectroscopy in photodissociation experiments, polarized single crystal absorption spectroscopy, and molecular dynamics simulations are being used to investigate structure function relations in myoglobin and hemoglobin. The technique of high-precision nanosecond spectroscopy in partial photolysis experiments has been developed and has led to several important new findings, including (i) demonstration that the transition state for the quaternary structural change of hemoglobin is much more like the R conformation than the T conformation, a result which explains the linear free energy relation between the quaternary rates and equilibrium constants, (ii) discovery of the protein relaxation subsequent to photodissociation of the myoglobin carbon monoxide complex that may be responsible for a time-dependent barrier to ligand rebinding, (iii) demonstration in hemoglobin that there is no communication between subunits prior to the quaternary conformational change at about 10 microseconds, (iv) and confirmation of theoretical predictions for the dependence of the optical anisotropy on the degree of photolysis with linearly-polarized light pulses.

Polarized single crystal absorption measurements show that crystals of hemoglobin in the T quaternary structure bind oxygen noncooperatively with no Bohr effect. These results provide strong evidence for the two-state allosteric model and for Perutz's stereochemical mechanism of the Bohr effect.

Studies of the domain structure and kinetics of hemoglobin S gel formation support the hypothesis that homogeneous nucleation of a single polymer molecule can trigger the formation of an entire domain of polymers, and that variations in the rate of homogeneous nucleation can account for the wide variety of shapes for sickled cells.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29011-20-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The physics and chemistry of photoreceptio

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. : William A. Hagins Medical Officer LCP-NIDDK

Others: S. Yoshikami, Research Biologist, LCP-NIDDK; P. Ross, Research Chemist, LMB-NIDDK; K. Spring, Research Med. Officer, LKM-NHI; Mark Vivino, Computer Systems Analyst, CSL-DCRT; Richard Leapman Research Physicist, BEIB-DRS; Lewis Pannell, Research Chemist, LAC-NIDDK

COOPERATING UNITS *(if any)*

Sudhir Sadhu, Student Volunteer, Thomas Jefferson High School, Alexandria, VA

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Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Improvements and extensions of pyroelectric calorimetry for study of heat production by milligram weight biochemical and cellular systems are under development.

Pyroelectric calorimetric studies of phototransduction in frog retinas are being extended to low light exposures and to retinas that have been subjected to sudden changes in their fluid environment.

A series of new compounds have been synthesized to be useful as elemental standards for estimating water content and transmembrane potentials in retinal and brain cells. Distributions of these compounds is being studies by electron energy-loss spectrometry.

The metabolism of esterified membrane-permeable fluorescent calcium indicator dyes is being studied by high-pressure liquid chromatography and mass spectrometry, with the aim of explaining the uneven cellular distribution of the dyes and the presence of fluorescent but calcium-insensitive forms in retinal photoreceptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-29016-15-LCP

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Macromolecular dynamics and assembly reactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	James Hofrichter	Research Chemist	LCP-NIDDK
Others:	William A. Eaton	Medical Officer	LCP-NIDDK
	Eric Henry	Research Chemist	LCP-NIDDK
	Marius Clore	Research Chemist	LCP-NIDDK
	Richard Lozier	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen Jones	Visiting Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

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Laboratory of Chemical Physics

SECTION
Section on Spectroscopy and Structure

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.7	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Time-resolved absorption spectroscopy is used to study the **dynamics** of **protein structural changes** subsequent to excitation with short laser pulses. Molecular models for the protein dynamics are used to fit and interpret the measured data.

A. The kinetics of ligand binding and conformational changes for **human hemoglobin** (HbA) have been studied following the **photodissociation of carbon monoxide** from the hemes. Analysis of a comprehensive set of data from partial photolysis experiments using polarized excitation has permitted cooperative and noncooperative processes to be clearly distinguished. We are now proceeding to test specific **molecular models** for the rebinding dynamics.

B. The photocycles of **bacteriorhodopsin (bR)**, a photoactive proton pump have been investigated. A detailed analysis of data collected by Xie et al. has shown that **back reactions** are significant for all steps of the cycle except the last. The temperature- and pH-dependence of these rates is obtained from fits of the model to the data.

C. We have explored the properties of **Singular Value Decomposition** (SVD) as a tool for the analysis of sets of time-resolved spectra. By using simulated data and carrying out SVD of statistically large numbers of data sets, we have been able to characterize the ability of SVD to average and reject noise.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-29017-12-LCP

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line betw the borders.)*
Spectroscopic investigation of membrane li , and models

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Ralph G. Adams	Research Physicist	LCP-NIDDK
Others:	Ira W. Levin	Research Chemist	LCP-NIDDK

COOPERATING UNITS *(if any)*

Sherwin Strauss (FDA)

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Laboratory of Chemical Physics

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NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS 1.5	PROFESSIONAL: 1.5	OTHER.

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using a variety of vibrational spectroscopic techniques, we have investigated the role of phospholipid, principally dipalmitoylphosphatidylcholine (DPPC), in human lung surfactant as an enhancer of alveolar stability during respiration. Because of the high fluorescence associated with pulmonary surfactant, we must use Fourier transform (FT) Raman spectroscopic approaches coupled with near-infrared laser excitation in order to obtain useful spectra of native material.

Although we have characterized the gel to liquid crystalline phase transition properties of DPPC and related systems from FT-Raman spectra, we have not yet discerned the dominant lipid/protein interactions present in lung surfactant. We have demonstrated, however, that FT-Raman techniques can provide an important new biophysical tool.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29019-11-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Theoretical studies on the dynamical aspects of macromolecular function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	A. Szabo	Research Chemist	LCP-NIDDK
Others:	X. Zhou	Research Fellow	LCP-NIDDK

COOPERATING UNITS *(if any)*

D. Dlott, University of Illinois; R. Zwanzig, LCP/NIDDK, A. Ansari, LCP/NIDDK

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NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The theory of geminate and bimolecular diffusion influenced reactions was used to interpret the rebinding of carbon monoxide to protoheme following photodissociation measured over ten decades of time (1ps to 10ms). It was shown that the highly nonexponential behavior of the initial geminate phase, which cannot be explained using simple kinetic schemes, is a reflection of the diffusive motion of the reactants. Molecular dynamics simulations have been used to obtain the kinetics of a simple model of fluorescence quenching in a hard sphere liquid. These computer generated "experimental" results we then used to test the Smoluchowski theory of irreversible reactions. Considering the many particle nature of the problem, the agreement between theory and simulations was surprisingly good over a wide range of quencher concentrations. The Smoluchowski theory has been generalized to treat second order reversible diffusion-influenced bimolecular reactions. It was shown that a small initial deviation of the concentrations from their equilibrium values decays to zero nonexponentially in contrast to the prediction of ordinary chemical kinetics. A theory was developed to obtain the time dependent rate of binding of ligands to a cell partially covered by receptors. A special case of the result derived in this work was used to find an analytic expression for the current at a random array of microdisk electrodes. Finally, a new brownian dynamics algorithm was developed to simulate the time dependent bimolecular rate coefficient for reactions involving species with complex geometries that interact via an arbitrary potential.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29020-07-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line bet[illegible] the borders)*

Nuclear magnetic resonance: new method[illegible]d molecular structure determination

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)*

P.I.: Ad Bax, Visiting Scientist, LCP-NIDDK

Others: Rolf Tschudin, Electronics Engineer; Mitsuhiko Ikura, Visiting Associate; Lewis Kay, Special Volunteer; Guang Zhu, Student Volunteer; Silvia Spera, Special Volunteer; Stephan Grzesiek, Special Volunteer; Gaetano Barbato, Special Volunteer: Frank Delaglio, Special Expert, Tony Sheng, Biological Aide; All LCP-NIDDK

COOPERATING UNITS *(if any)*

Marius Clore, Angela Gronenborn, NIDDK/LCP, Dennis A. Torchia, NIDR/LBR; Claude Klee, NCI/LB.

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Laboratory of Chemical Physics

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Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
7.5	5.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

Approaches developed in the previous year for making resonance assignments in larger proteins have been extended and improved. The new methods are affected to a lesser degree by the large resonance line widths which are approximately proportional to the molecular weight of the protein. A four-dimensional NMR experiment has been developed that dramatically reduces spectral overlap in one of the most crowded regions of the NOE spectrum, now permitting the study of interactions between aliphatic residues in proteins of a substantial size. The new experiments provide access to a large number of parameters, such as ^{13}C and ^{15}N chemical shifts and coupling constants, and comparison with crystallographic data indicates that these parameters contain structurally important information. The new techniques have been applied to the study of the interaction between calmodulin and a 26-residue fragment of myosin light chain kinase. A dramatic change in the relative orientation of the two domains of calmodulin upon complexation with the peptide is observed and the peptide changes from a random coil to an alpha-helical conformation upon complexation. A detailed structural characterization is currently in progress.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29021-06-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Conformation and dynamics of biological macromolecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric R. Henry	Research Physicist	LCP-NIDDK
Others:	William A. Eaton	Medical Officer	LCP-NIDDK
	James Hofrichter	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen M. Jones	IRTA Fellow	LCP-NIDDK
	Attila Szabo	Research Chemist	LCP-NIDDK

COOPERATING UNITS *(if any)*

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, Italy

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have studied in detail the properties of the singular value decomposition of spectroscopic data sets which contain different types of measurement noise. The effects of totally random or "shot" noise on the SVD are qualitatively different from those of "wavelength-correlated" or baseline noise, and it is possible by post-processing the SVD to recover most of the signal content from a noisy data set only in the latter case. We have also characterized both experimentally and theoretically the effects of rotational diffusion on transient absorption measurements made with polarized photolysis and probe laser pulses. Procedures have been developed for obtaining properly isotropically averaged data sets from such measurements, which should allow determination of the conformational and ligand binding kinetics of hemoglobin with unprecedented accuracy. We have also derived oxygen binding curves for single crystals of human hemoglobin from polarized optical absorption spectra of crystals at various oxygen pressures. Hemoglobin in the crystal binds oxygen essentially non-cooperatively and with very low affinity, and these binding properties are essentially independent of pH (i.e., there is no Bohr effect). We have also performed large molecular dynamics simulations of the cooling of the photoexcited heme group in myoglobin in solution, and found that the dissipation of the excess heme energy in the protein in solution takes place only slightly faster than in the protein in a vacuum. We are also performing very long simulations of myoglobin in vacuo in order to study the orientational dynamics of the heme group in this protein to aid in the interpretation of anisotropy data from experiments using polarized excitation and probe pulses.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-29022-04-LCP

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structural studies of AIDS proteins by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Ad Bax	Visiting Scientist	LCP-NIDDK
Others:	Jim Omichinski	Staff Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK
	Dan Garrett	IRTA Fellow	LCP-NIDDK
	Bruce Grasberger	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)
Protein Expression Laboratory, OD (Stephen Stahl, Paul Wingfield); NCI (Ettore Appella, Sam Wilson and Pat Becerra)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3	3	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work has been carried out on a number of structural problems related to proteins derived from the HIV virus. These include the RNAse H domain of HIV-1 reverse transcriptase, the p7 nucleocapsid protein of HIV-1, and proteins of the immune system, in particular interleukin-1β and the double zinc finger domain of the human enhancer binding protein. The secondary structure of the RNaseH domain has been determined using double and triple resonance 3D NMR spectroscopy, and current work is being focused on studying its internal dynamics and obtaining a three-dimensional structure in solution. The solution structure of the zinc finger domains of the p7 nucleocapsid protein has been determined, and the two zinc binding domains shown to be identical within the errors of the coordinates. The high resolution structure of interleukin-1β in solution has been determined using 3D and 4D heteronuclear NMR. In addition, structural studies have begun on the double zinc finger domain of the human enhancer protein which binds specifically to the regulatory region in the long terminal repeat of the HIV genome.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29023-04-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Determination of Three-Dimensional Structures of Macromolecules in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK

Others: Jim Omichinski, Staff Fellow, LCP-NIDDK; Julie Forman-Kay, IRTA Fellow, LCP-NIDDK; Dan Garrett, IRTA Fellow, LCP-NIDDK; Bruce Grasberger, IRTA Fellow, LCP-NIDDK; Robert Powers, IRTA Fellow, LCP-NIDDK; Mark Robien, Guest Researcher, LCP-NIDDK

COOPERATING UNITS (if any)

Laboratory of Chemical Physics, NIDDK (Ad Bax), Protein Expression Laboratory (Stephen Stahl, Paul Wingfield); NCI (Ettore Appella); Genex Corp. (Marc Whitlow), Univ. South Dakota (Peanasky)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work in this laboratory has been focussed on the determination of three-dimensional structures of larger proteins in solution by NMR, with a particular emphasis on cytokines and immune related proteins. A considerable effort has been placed on the development of three- and four-dimensional heteronuclear NMR which offers dramatic improvements in spectral resolution by spreading through-bond and through-space correlations in three and four orthogonal frequency axes. Simultaneously, large heteronuclear couplings are exploited to circumvent problems due to larger linewidths that are associated with increasing molecular weight. These novel experiments have been specifically designed to extend the application of NMR as a method for determining three-dimensional structures of proteins in solution beyond the limits of conventional two-dimensional NMR (~100 residues) to molecules in the 150- to 300-residue range. This potential has recently been confirmed by the determination of the first high resolution NMR structure of a protein greater than 150 residues, namely interleukin-1β.

High resolution solution structures of a number proteins have been determined. These include the cytokine interleukin-1β and the IgG binding domain of protein G from Streptococcus. Extensive use in these studies has been made of multi-dimensional heteronuclear NMR and of systematic conformational searches to obtain stereospecific assignments and torsion angle restraints which have enabled us to obtain very high resolution structures comparable in accuracy to 2 Å resolution X-ray structures. The typical accuracy attainable is 0.2-0.4 Å for the backbone atoms and 0.4-0.5 Å for the internal side chains.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29025-03-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Investigations of Macromolecular Structures and Dynamics in Solution by NMR

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK

Others: Jim Omichinski, Staff Fellow, LCP-NIDDK; Julie Forman-Kay, IRTA Fellow, LCP-NIDDK; Dan Garrett, IRTA Fellow, LCP-NIDDK; Bruce Grasberger, IRTA Fellow, LCP-NIDDK; Robert Powers, IRTA Fellow, LCP-NIDDK; Mark Robien, Guest Researcher, LCP-NIDDK

COOPERATING UNITS *(if any)*

Laboratory of Chemical Physics, NIDDK (Ad Bax), Protein Expression Laboratory, OD (Stephen Stahl, Paul Wingfield); NCI (Ettore Appella); Genex Corp. (Marc Whitlow).

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of the overall research in this laboratory is centered on achieving as complete a description as possible for the structures of peptides, proteins, nucleic acids and their complexes in solution, pricipally by NMR spectroscopy. At present particular emphasis is placed on developing approaches which allows the investigation of larger systems as well as increase the precision with which these solution structures can be obtained.

Structures for several proteins have been determined and analyzed. These include the cytokine interleukin-1β which, at 153 residues, is 1.5 times larger than the largest protein structure previously solved by NMR spectroscopy, the IgG binding domain of protein G from Streptococcus, and human thioredoxin. These solution structures represent very high resolution structures, with errors in the atomic coordinates of 0.2-0.4 Å for the backbone atoms, and 0.4-0.5 Å for ordered side chains. These studies have exploited many novel 3D and 4D heteronuclear NMR experiments to dramatically increase spectral resolution and thereby resolve assignment ambiguities in larger proteins. Of particular interest in structural terms is the finding that the IgG binding domain of protein G has a novel polypeptide fold which has heretofore not been observed and which may account for its unusually high thermal stability.

Work is in progress on several other proteins, such as the extracellular domain of the interleukin-1 receptor, the cytokine interleukin-4, the double zinc finger domain of the human enhancer binding protein, the RNase H domain of HIV-1 reverse transcriptase, the nucleocapsid protein of HIV-1, the A1 heteronuclear RNA binding protein, the DNA binding protein ner from phage Mu and Ascaris trypsin inhibitor.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-29026-03-LCP

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
NMR and Other Spectroscopic Studies of Molecular Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. :	Edwin D. Becker	Research Chemist	LCP-NIDDK

COOPERATING UNITS *(if any)* Sophisticated Instruments Facility, Indian Institute of Science, Bangalore, India; Centre for Cellular and Molecular Biology, Hyderabad, India; Rudjer Boskovic Institute, Zagreb, Yugoslavia [all foreign]

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
NMR Section

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.7	0.7	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A. Collaborative studies with C. L. Khetrapal and others at the Indian Institute of Science use nuclear magnetic resonance (NMR) methods to study molecules oriented in liquid crystals. Because the spectra rapidly become very complex with increasing numbers of interacting hydrogen atoms, only small or highly symmetric molecules have been studied. We are exploring a new method to use solid-state NMR spinning techniques to extend the range of applicability of the method.

B. A new analysis of the fundamental vibrations of p-benzoquinone has been carried out, using recently acquired Raman spectroscopic data. Several confusing aspects of the assignment have now been explained satisfactorily.

C. In collaboration with D. Vikic-Topic and others at the Rudjer Boskovic Institute, we are exploring the mechanism and potential applications of the effect of isotopic substitution on NMR chemical shifts, as shown in nuclei up to 10 bonds away from the site of substitution.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29027-03-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Theoretical studies of dynamical processes [illegible] chemical physics and biophysics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. :	Robert Zwanzig	Research Chemist	LCP-NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysics Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the last year, the main focus of research has been on studies of diffusion influenced reactions. One investigation led to a new time dependent version of Berg and Purcell's steady state theory of diffusion influenced ligand binding to receptors randomly distributed on the surface of a cell. The results may be useful, for example, in treating the dynamics of the infection of a cell culture by a virus. Another application of the general theory was to work out the time dependent current to a random ensemble of microdisk electrodes. This is of considerable interest in electroanalytical chemistry. In a second investigation, theory and computer simulations were applied to a model for a chemical reaction whose rate is controlled by an entropy barrier rather than the more common potential barrier. The ideas developed here may be useful in understanding the dynamics of the escape of ligands from the heme pocket on myoglobin and hemoglobin. In a third investigation, attention is given to the general theory of reversible diffusion influenced bimolecular reactions. Considerable progress has been made in formulating a reliable theoretical approach, and this approach is being tested by computer simulations on a simple model.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29028-01-LCP

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Free Energy Conversion in Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Yi-der Chen	Research Chemist	LCP-NIDDK
Others:	Robert J. Rubin	Special Volunteer	LCP-NIDDK

COOPERATING UNITS *(if any)*

Shahid Khan, Dept. of Physiology & Biophysics, Albert Einstein College of Medicine, NY; Joe Chalovich, Dept. of Biochemistry, East Carolina University School of Medicine, Greenville, NC

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysics Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A number of different topics have been studied in the general field of free energy transduction and biophysics of biological systems. The most important areas in which progress has been made are the study of dynamics of flagellar motor rotation in bacteria, the dequenching kinetics of fluorescence of lipid-like probes in membrane fusion systems, the equilibrium binding of caldesmon molecules to actin in the presence of myosin molecules, and one-dimensional random walks in the presence of traps.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—564 939

ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research of the Laboratory is directed towards the introduction of new concepts, techniques and agents for the elucidation of the molecular nature of mechanisms controlling cell functions. Specific focus is placed on i) Development of selective agonists/antagonists for receptors controlling cyclic nucleotide formation, phospholipid metabolism and ion channel function; ii) The relationship between ion transport, phospholipid turnover and cyclic nucleotide generation and the delineation of agents with specific effects on macromolecules involved in these systems. iii) The isolation and structure elucidation of biologically active natural products and definition of the basis of their activity. iv) Effects of agents on ion channels and the development of radioactive ligands for modulatory sites in such channels. v) The nature of enzymes involved in formation and inactivation of neurotransmitters, hormones, and other modulatory substances, in particular the enzymes, catechol-O-methyltransferase, monoamine oxidase, adenylate cyclase and phosphodiesterases. vi) the fundamental mechanisms by which drugs and environmental chemicals are transformed in the body with emphasis on oxidative metabolism by cytochrome P-450 systems to generate active oxide metabolites that interact wtih macromolecules such as DNA and are metabolized by further oxidation, by hydrolysis and by conjugation with glutathione.

Some of the milestones for the Laboratory are as follows: i) Introduction of the adenine-prelabeling technique for study of cyclic AMP generation in intact cells; ii) The steroidal alkaloid batrachotoxin as a selective activator of sodium channels. iii) Histrionicotoxin as a noncompetitive blocker of acetylcholine receptor channels and potassium channels. iv) Pumiliotoxins as myotonic and cardiotonic alkaloids acting through sodium channels to elicit phosphoinositide turnover. v) N^6-Substituted adenosines and 8-phenyl and 8-cyclohexylxanthines and other heterocycles as selective and potent adenosine receptor agonists and antagonists suitable as radioligands for binding studies and for definition of A_1 and A_2 classes of receptors. vi) Introduction of forskolin as a specific and widely useful activator of adenylate cyclase. vii) Fluoronorepinephrines and analogs as selective alpha and beta-adrenergic agonists. viii) Production of antibodies to catechol-O-methyl transferase and their use in studying localization of this key catechol-metabolizing enzyme. ix) Development of a cyclic quaternary amine, isoarecolone methiodide, with high potency and selectivity for nicotinic receptors. x) Definition of a relationship between receptor-activation of phosphoinositide breakdown; protein kinase C activation, and altered responses of cyclic AMP-generating systems. xi) Introduction of maitoxin as a general activator for phosphoinositide breakdown. xii) Discovery of the NIH shift of aryl substituents during P-450 catalyzed phenol formation and demonstration of arene oxides as intermediates. xiii) Demonstration of oxidation-hydrolysis pathways that convert stereoselectively polycyclic aromatic hydrocarbons to ultimate diol epoxides that react with DNA. xiv) Discovery and formulation of the bay-region theory, which is predictive of the pathway for formation of reactive carcinogenic metabolites from polycyclic aromatic hydrocarbons. xv) Development of optical assays for protease and reverse transcriptase of HIV-1.

The laboratory accomplishes its mission both through its own resources and through extensive collaborations with other laboratories both at NIH, at Universities, Museums, and other institutes and in drug and chemical companies. Such collaborations can involve sharing of expertise on syntheses, isolations, analyses and biological testing and field work to obtain sources of new natural products.

SECTION ON PHARMACODYNAMICS

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

Structure elucidation of alkaloids from skin of frogs and toads continues to reveal unique new compounds many with high biological activity. Syntheses for several of these are under investigation, including the pseudophrynamines, which based on structural analogy to physostigmine, may be acetylcholine esterase inhibitors, epibatidine, a novel pyridyl-nortropane with potent non-opioid analgetic activity and the pyrrolidine oximes. Tetrodotoxin-like compounds, cannabinoid-like compounds, and ouabain-like compounds have been identified in extracts from amphibian skin based on antagonism of binding of radioligands. The ouabain-like compounds proved to be bufodienolides and unexpectedly also cardenolides in one genus (Atelopus) of bufonid toads. Derivatives of aconitine have been prepared, but none have proven significantly more potent than aconitine at voltage-dependent sodium channels and, thus, will not serve as radioligands. Other sources of biologically active compounds are under investigation including a toxic bird and plant extracts used in folk medicine. Investigation of mechanisms underlying the biological activity of alkaloids and other natural products continues, but in the case of the pseudophrynamines, the analgetic epibatidine, and the pyrrolidine oximes this is dependent on current synthetic efforts.

A NEW CLASS OF ALKALOIDS FROM A DENDROBATID FROG: A STRUCTURE FOR ALKALOID 251F. Alkaloids of a new class are present in skin extracts of the dendrobatid poison frog Minyobates bombetes of Colombia. The structure of the major alkaloid of this class, 251F, has been determined as a trimethylcyclopenta[b]quinolizidinemethanol by NMR, GC-FTIR and mass spectral studies including MS-MS. At least nine congeners of 251F were detected in these extracts, in addition to alkaloids of the pumiliotoxin-A class and 5,8-disubstituted indolizidine and 1,4-disubstituted quinolizidine alkaloids.

PYRROLIZIDINE OXIMES: A NOVEL NEW CLASS OF DENDROBATID ALKALOIDS. Analysis of GC-FTIR spectra and reexamination of ^{1}H- and ^{13}C-NMR data led to revised structures for three closely related tricyclic alkaloids from a dendrobatid poison-frog Dendrobates pumilio. The simplest member 222 is a 9,9-dimethyl[4.4]spiropentano-pyrrolizidine-4-oxime, while 236 is a corresponding O-methyl oxime and 252 is a 3-hydroxy-O-methyl oxime.

FURTHER PUMILIOTOXINS AND ALLOPUMILIOTOXINS AND A REASSIGNMENT OF THE KETO FUNCTION IN PUMILIOTOXIN 307F. Skin extracts from the Panamanian poison-frog Dendrobates pumilio have afforded further trace alkaloids of the pumiliotoxin A (PTX-A) class (6-alkylidene-8-hydrozy-8-methylindolizidines) and an allopumiliotoxin subclass (7-hydroxy-PTX-A). Structures for pumiliotoxins 209F and 225F and allopumiliotoxin 225E, all with four carbon 6-alkylidene side chains, for allopumiliotoxins 309D, 325A′ and 325A", all with ten carbon 6-alkylidene side chains, and a reassignment of the position of the keto function in the ten carbon 6-alkylidene side chain of pumiliotoxin 307F have been determined. Carbon-13 magnetic resonance assignments have been made for these and the 15-O-methyl ether artefacts of pumiliotoxin 307A and allopumiliotoxin 323B.

FURTHER CIS-DECAHYDROQUINOLINES AND 8-METHYLINDOLIZIDINES. Skin extracts from one population of the poison frog Dendrobates auratus contain a variety of alkaloids, including 2,5-disubstituted-cis-decahydroquinolines and 5-substituted-8-methylindolizidines. Three of the major alkaloids are cis-decahydroquinolines, whose structures based on NMR analyses are 2,5-diallyl-cis-decahydroquinoline (cis-219A), 2-allyl-5(pent-2-en-4-ynyl)-cis-decahydroquinoline (cis-243A) and 5-methyl-2-propyl-cis-decahydroquinoline (cis-195A); the last is identical with "pumiliotoxin C" previously isolated from the poison frog Dendrobates pumilio. Alkaloids cis-219A and cis-243A differ from cis-195A in configuration at the C-2 position. Another poison frog, Dendrobates histrionicus, was previously shown to produce nearly exclusively trans-decahydroquinoline isomers of 219A and 243A. NMR analyses indicate that the structure of a minor alkaloid from Dendrobates auratus is 5-(pent-2-en-4-ynyl)-8-methylindolizidine (203A). Two additional 8-methylindolizidines isolated from Dendrobates pumilio are 5-(hept-4,6-dienyl)-8-methylindolizidine (233D) and 5-(hept-6-hydroxy-4-enyl)-8-methylindolizidine (251B). FTIR spectral analyses distinguish cis- and trans-decahydroquinolines and provide evidence for configurations of indolizidines.

FROG SECRETIONS AND HUNTING MAGIC IN THE UPPER AMAZON: IDENTIFICATION OF A PEPTIDE INTERACTING WITH ADENOSINE RECEPTOR. A frog used for "hunting magic" by several groups of Panoan-speaking Indians in the borderland between Brazil and Peru is identified as Phyllomedusa bicolor. This frog's skin secretions, introduced to the body through fresh burns, are rich in peptides. These include vasoactive peptides, opioid peptides, and a new peptide--adenoregulin--for which amino acid analysis indicates a minimum molecular weight of 2871. Adenoregulin enhances binding of agonists to A_1-adenosine receptors; it is accompanied by peptides that inhibit binding. The sequence of adenoregulin has been determined and investigation of its biological properties will be continued with synthetic material.

ALKALOID PRODUCTION IN DENDROBATID FROGS. VARIABILITY IN ALKALOID PROFILES IN NEOTROPICAL POISON FROGS (DENDROBATIDAE): Dendrobatid frogs produce a diverse set of alkaloids, whose profiles appear characteristic of frogs of each species or, in the case of variable species, of each population. In the case of one wide-spread species, Dendrobates auratus, alkaloid profiles in extracts of skin are markedly different in three populations, one from a small Pacific island (Isla Taboga, Panama) one from central mountains in Panama, and the third from the Caribbean coast in Costa Rica. The first contains three major classes of dendrobatid alkaloids, the histrionicotoxins, the pumiliotoxin-A class and the decahydroquinolines. The second contains mainly histrionicotoxins and the pumiliotoxin-A class alkaloids. The third contains histrionicotoxins, a homopumiliotoxin, one decahydroquinoline, and a variety of indolizidines, quinolizidines and pyrrolizidines. Frogs from Isla Taboga were introduced into the Manoa Valley, Oahu, Hawaii in 1932. Remarkably, although alkaloids of the pumiliotoxin-A class are still major constituents in skin extracts of Hawaiian frogs descended from the 1932 founding population, histrionicotoxins and decahydroquinolines are absent and a novel tricyclic alkaloid is present. Offspring of wild-caught parents from Hawaii, Panama or Costa Rica raised in indoor terrariums on a diet of crickets and fruit flies do not contain detectable amounts of skin alkaloids. Offspring of Hawaiian frogs raised in large outside terrariums in Hawaii and fed mainly wild-caught termites and fruit flies do contain the same profile of alkaloids as their wild-caught parents in Hawaii, but at reduced levels. The genetic, environmental and dietary determinants of alkaloid profiles in dendrobatid frogs remain obscure.

NICOTINIC RECEPTOR-ELICITED SODIUM FLUX IN RAT PHEOCHROMOCYTOMA PC12 CELLS: EFFECTS OF AGONISTS, ANTAGONISTS, AND NONCOMPETITIVE BLOCKERS. Nicotinic agonists stimulate ^{22}Na flux in rat pheochromocytoma PC12 cells. The stimulatory effect of carbamylcholine is maximal at 1 mM, while the stimulatory effect of nicotine and anatoxin maximize at the same level at 100 μM and 10 μM, respectively. The tertiary amines arecolone and isoarecolone have no effect on flux at 100 μM, while the methiodides at 100 μM stimulate flux to an extent similar to 1 mM carbamylcholine. A preincubation for 2 to 20 min with carbamylcholine (2 mM), nicotine (300 μM), anatoxin (30 μM) or isoarecolone methiodide (100 μM) causes marked desensitization to a subsequent carbamylcholine-elicited stimulation of flux. d-Tubocurarine, mecamylamine, hexamethonium, and chlorisondamine inhibit carbamylcholine-elicited flux with IC_{50} values of 1.0, 0.8, 43 and 0.020 μM, respectively. Atropine has no effect at 1 μM, but reduces the response to carbamylcholine by 50% at 8.6 μM, presumably as a noncompetitive blocker. Other noncompetitive blockers of nicotinic acetylcholine-receptors, such as histrionicotoxins, gephyrotoxin, pumiliotoxin C, phencyclidine, bupivacaine and piperocaine, inhibit carbamylcholine-elicited stimulation of ^{22}Na flux with IC_{50} values from 0.3 to 1.8 μM. In contrast to d-tubocurarine, which inhibits carbamylcholine-elicited desensitization, and mecamylamine, which has no apparent effect on desensitization, chlorisondamine and certain noncompetitive blockers appear to enhance desensitization. The effects of agonists, antagonists and noncompetitive blockers at the neuronal nicotinic acetylcholine receptor-channel of PC12 cells are compared to their effects on binding of [^{125}I]α-bungarotoxin to agonist-recognition sites and of [^{3}H]perhydrohistrionicotoxin to noncompetitive blocker sites of the nicotinic acetylcholine receptor-channel of electric ray (Torpedo) electroplax membranes. There are marked differences for certain blockers in relative potencies for the two types of nicotinic acetylcholine receptor-channel. The cis- and trans-decahydroquinolines represent a class of noncompetitive blockers for acetylcholine receptor-channels with similar activity for both muscle-type and ganglionic type nicotinic receptor-further channels.

5,8-DISUBSTITUTED INDOLIZIDINES: A NEW CLASS OF NONCOMPETITIVE BLOCKERS FOR NICOTINIC RECEPTOR-CHANNELS. A series of 8-methyl-5-substituted indolizidines inhibit binding of the noncompetitive blocking agent [^{3}H]perhydrohistrionicotoxin to muscle-type nicotinic acetylcholine receptor-channels in membranes from Torpedo electroplax. The Ki values range from 0.16 to 1.12 μM, making these alkaloids among the most potent ligands for this site. Unlike most noncompetitive blockers, the potencies of the 8-methyl-5-substituted indolizidines are reduced in the presence of carbamylcholine. Indolizidine 205A (8-methyl-5-(4-pentynyl)-indolizidine) is unique in enhancing binding of [^{3}H]perhydrohistrionicotoxin by 1.5-fold. The enhancement is at a maximum at 0.01 to 0.1 μM, followed by progressive inhibition with an IC_{50} of about 20 μM. In the presence of carbamylcholine, which itself enhances binding of [^{3}H]perhydrohistrionicotoxin, indolizidine 205A causes only an inhibition of binding with an IC_{50} of about 10 μM. Indolizidines with a hydroxy substituent on the 8-methyl group have very low activity. None of the indolizidines affect binding of [^{125}]α-bungarotoxin to acetylcholine recognition sites. In pheochromocytoma PC12 cells, indolizidine 205A has no agonist activity, but only inhibits carbamylcholine-elicited $^{22}Na^{+}$ influx. The profile of potencies for the 8-methyl-5-substituted indolizidines is similar in electroplax membranes and PC12 cells. Indolizidines 205A and 209B (8-methyl-5-pentylindolizidine) have no apparent effect on desensitization of receptors in PC12 cells. The 5,8-disubstituted indolizidines appear to represent an atypical and potent class of noncompetitive blockers for muscle-type and ganglionic nicotinic receptor-channels.

INTERACTION OF PUMILIOTOXIN B WITH AN "ALKALOID-BINDING DOMAIN" ON THE VOLTAGE-DEPENDENT SODIUM CHANNEL. The alkaloid pumiliotoxin B (PTX-B) "activates" voltage-dependent sodium channels in synaptoneurosomes and neuroblastoma cells. It appears that PTX-B activates sodium channels by interacting with a site that is allosterically regulated by other sites, namely the scorpion toxin sites and the brevetoxin site, on the sodium channel. In guinea pig cortical synaptoneurosomes, *α*-scorpion toxin, ß-scorpion toxin and the marine toxin brevetoxin induce a dose-dependent potentiation of PTX-B-induced $^{22}\mathrm{Na}^+$ influx. The synergism with ß-scorpion toxin differentiates PTX-B from the alkaloids batrachotoxin and veratridine, which induce an activation of sodium channels that is not affected by ß-scorpion toxin. PTX-B does not inhibit [^{3}H]batrachotoxinin-A benzoate ([^{3}H]BTX-B) binding to the alkaloid site sodium channels. On the other hand, aconitine, which activates sodium channels and inhibits [^{3}H]BTX-B binding, induces a $^{22}\mathrm{Na}^+$ influx that, like PTX-B-induced $^{22}\mathrm{Na}^+$ influx, is potentiated by *α*-scorpion toxin, ß-scorpion toxin and brevetoxin. Inhibition of [^{3}H]BTX-B binding by aconitine is reduced in the presence of PTX-B. The type I pyrethroid allethrin, but not the type II pyrethroid deltamethrin, inhibits PTX-B- and PTX-B/*α*-scorpion toxin-mediated $^{22}\mathrm{Na}^+$ flux, but not BTX- and veratridine-mediated $^{22}\mathrm{Na}^+$ influx. It is proposed that on the sodium channel there is an "alkaloid binding domain" at which alkaloids exert stimulatory actions. However, depending on the region on the domain on which the binding occurs, different allosteric actions with other sites can be observed. PTX-B is proposed to interact with a part of the "alkaloid binding domain" that is shared by aconitine, but not by batrachotoxin or veratridine, while aconitine interacts with a part of the domain shared by PTX-B and by batrachotoxin/veratridine.

SECTION ON PHARMACODYNAMICS

Pharmacology and Metabolism of Biogenic Amines and Related Compounds.

COMT: Light and electron-microscopic immunocytochemical observations of COMT in rat uterus and other tissue were made with a specific antibody to the soluble form of rat liver COMT and peroxidase conjugated with streptavidin. These studies have included the demonstration that the appearance of COMT in the luminal epithelium and glandular epithelium of the metrial gland of rat in very early pregnancy and pseudopregnancy is a progesterone-dominated phenomena. Present studies center on the effect of progesterone-receptor inhibitors on the appearance of luminal COMT: the demonstration that COMT in the dental pulp of rat is present in macrophages; and the demonstration that COMT and macrophage-specific antigens are located in macrophages of the corpus lutea of rat. In addition COMT activity is also being examined in kidneys following estrogen-induced renal carcinogenesis in the hamster and in estrogen receptor-positive and -negative endrometrial and breast adenocarcinomas. Studies on the role of COMT in biosynthesis of mammalian opioids have included the O-methylation of (S)- and (R)-3′-hydroxycoclaurines and (S)- and (R)-3′-hydroxy-N-methylcoclaurines. The (S)- and (R)isomers of the nor-series afforded almost equal amounts of norreticulines and nororientalines, and two additional unknown products. The (S)-N-methylated isoquinoline, on the other hand, afforded largely (S)orientaline, and an almost equal mixture of (R)-reticuline and (R)-orientaline. The nature of the unknown O-methylated products derived from both isomers of the nor-series is being investigated.

Fluorine and Other Derivatives of Biogenic Amines and Amino Acids: The effect of 2-azidotyrosine on the incorporation of isotopically labelled tyrosine and leucine into the proteins of PC12 cells in culture was examined in the presence and absence of NGF. 2-Azidotyrosine at a maxium concentration of 0.1 mM did not block the incorporation of leucine in PC12 protein over a period of 5 days. However 2-azidotryosine inhibited the incorporation of tyrosine into PC12 cell protein in a time and dose dependent manner, a maxium inhibition of 58 percent of control was observed at a concentration of 0.01 mM after 72 hours of growth. 2-Azidotyrosine at 0.1 mM inhibited cell growth as measured by thymidine incorporation by 20-30 percent of control values. The present data suggest that 2-azidotyrosine is incorporated into PC12 cell protein in place of tyrosine.

PC12 Cell Studies: PC12 cells are a nerve growth factor-responsive clone derived from a rat pheochromocytoma. The cells contain catecholamine and secrete them in response to depolarizing stimuli and cholinergic agonists. Treatment of the cells with nerve growth factor produces a number of very rapid changes including the structural rearrangement of cell membrane, the generation of a number of different second messengers, and the phosphorylation of several proteins. Nerve growth factor treatment increases the release of dopamine and norepinephrine from the cells within a few minutes and does so independently of its effect on their metabolism. This effect of nerve growth factor is dependent on the presence of extracellular calcium and can be blocked by calcium channel antagonists. NGF also stimulates calcium ion uptake into cells attached to collagen-polylysine coated plates by 60-80% within 3 min., but this effect is trancient, being much lower or absent after 15 min of NGF treatment. The effect of NGF is blocked by EGTA, K-252b, staurosporin, calmidazolium, fluphenazine and suramine. The effect of NGF was not blocked by inhibitors of N- or T-type calcium channels nor by calsineurin, nickel, or inhibitors of protein kinase A, C, or N, and only partially with high concentrations of L-type channels. The NGF effect on calcium ion uptake was much less in cell suspension, in very dense culture, or in cells pretreated with calcium inophores. The data indicate that most of the NGF-stimulated increase in calcium ion uptake relies on channels that are different the well-characterized voltage- or receptor-operated calcium channels.

SECTION ON PHARMACODYNAMICS

Ion Channels, Receptors and Second Messengers in the Nervous System

MAITOTOXIN: SITE OF ACTION: The dinoflagellate toxin maitotoxin (MTX) elicited a sustained increase of $[Ca^{2+}]_i$ in rat C6 glioma cells. This response was inhibited by SK&F 96365, a blocker of receptor-mediated calcium entry. In C6 cells, endothelin-I elicited a large and rapid, but transient increase in $[Ca^{2+}]_i$, followed by a small, sustained increase and SK&F 96365 inhibited only the sustained increase in $[Ca^{2+}]_i$. In both C6 glioma cells and RIN insulinoma cells, MTX elicited a marked influx of $^{45}Ca^{2+}$. SK&F 96365 inhibited MTX-induced $^{45}Ca^{2+}$ influx by 95% at 30 μM. The L-type calcium channel blocker nifedipine at 10 μM inhibited MTX-induced calcium uptake by 20% in RIN cells and by only 10% in C6 cells. MTX elicited calcium-dependent phosphoinositide breakdown in both C6 and RIN cells. In both cell lines, the MTX-induced phosphoinositide breakdown was inhibited by SK&F 96365 by 90% at 30 μM. Endothelin-1 and carbamylcholine elicit phosphoinositide breakdown in C6 cells and RIN cells, respectively. The stimulations were unaffected by the presence of SK&F 96365 at concentrations up to 100 μM. In RIN insulinoma cells, MTX elicited calcium-dependent release of insulin. SK&F 96365 at 30 μM inhibited MTX-induced insulin release by 75%, while nifedipine at 30 μM inhibited release by only 10%. The results suggest that MTX increases intracellular calcium by interacting directly with a calcium-entry system normally activated by receptors that elicit phosphoinositide breakdown and blocked by SK&F 96365. Activation of phospholipase C and hormone release by MTX also were blocked by SK&F 96365 and may be dependent on the initial activation of such a calcium-entry system.

MAITOTOXIN: EFFECT OF ANALOGUES. MTX and the analogues, bis-desulfated-MTX (didesulfo-MTX), mono-desulfated MTX (monodesulfo-MTX), and hydrogenated-MTX (H-MTX) were examined on $^{45}Ca^{++}$ influx and phosphoinositide breakdown with hamster insulinoma HIT cells and rat glioma C6 cells. The activity of MTX was greatly reduced either by desulfation or by hydrogenation. Didesulfo-MTX weakly stimulated calcium influx in HIT cells, but had no stimulatory effect on either calcium influx or phosphoinositide breakdown in C6 cells. All the analogues inhibited MTX-induced calcium influx in either HIT or C6 cells. Didesulfo-MTX inhibited the calcium influx elicited by 3 ng/ml MTX in C6 cells with an IC_{50} of 7.0 ± 0.7 ng/ml. The data suggest thatthe sulfate groups in MTX are important for stimulation of calcium influx and phosphoinositide breakdown, but are not essential for binding to a recognition-site on cell membranes. Although catalytic reduction of double bonds in MTX reduced activity by nearly 100-fold, a tritiated H-MTX still represents a potential radioligand for identification of MTX-binding sites.

THE EFFECTS OF THE AMPHIPHILIC PEPTIDES MELITTIN AND MASTOPARAN ON CALCIUM INFLUX, PHOSPHOINOSITIDE BREAKDOWN AND ARACHIDONIC ACID RELEASE IN RAT PHEOCHROMOCYTOMA PC12 CELLS. Two amphiphilic peptides from hymenopterid insects, melittin and mastoparan, stimulate secretion in a variety of cell types. In PC12 cells, both peptides stimulate calcium influx with melittin some 20-fold more potent than mastoparan. Melittin stimulates both breakdown of phosphoinositides (PI) by phospholipase C (PLC) to yield inositol phosphates and hydrolysis of phospholipids by phospholipase A_2 (PLA_2) to release arachidonic acid (AA). Mastoparan stimulates PI breakdown, but has no effect on AA release. Maximal stimulation of PI breakdown occurs at 2.5 mg/ml melittin and 30 mg/ml mastoparan, while maximal stimulation of AA release occurs at 5 mg/ml melittin. Organic calcium channel blockers (nifedipine, verapamil, diltiazem) have little or no effect on responses to the peptides. The influx of calcium elicited by melittin or mastoparan is completely or nearly completely blocked by inorganic calcium channel blockers (Co^{2+}, Mn^{2+}, Cd^{2+}). Mn^{2+} and Cd^{2+} inhibit melittin-induced PI breakdown and AA release and mastoparan-induced PI breakdown. Co^{2+} has no effect on melittin-induced PI breakdown and potentiates mastoparan-induced PI breakdown. Pertussis toxin has no effect on the PI breakdown induced by either peptide.

PHOSPHOLIPASE D: SITE OF PHOSPHATE BOND CLEAVAGE. Mammalian phospholipase D has been implicated in signal-transduction mechanisms, most recently in association with stimuli that enhance phosphatidylcholine (PC) turnover. It was previously unknown whether hydrolysis of PC by phospholipase D proceeds via P-O or C-O bond cleavage. Commercially available phospholipase D isolated from Streptomyces chromofuscus was used to hydrolyse distearoyl phosphatidylcholine (PC) in a detergent-containing buffer consisting of 90% ^{18}O-water. The product of hydrolysis, phosphatidic acid (PA), was purified by thin-layer chromatography and analyzed using californium-252 plasma desorption mass spectrometry. An increase of two mass units was observed, compared to a distearoyl PA control, consistent with a reaction mechanism involving cleavage of the P-O bond.

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites.

Quinoline is a well recognized environmental carcinogen. However, little is known about the mechanism of its metabolic activation. As part of our approach to the study of this azapolycyclic hydrocarbon, we have developed synthetic methods for the preparation of optically active metabolites of known absolute configuration at the 5,6-double bond. Racemic *trans*-6-bromo-5-hydroxy-5,6,7,8-tetrahydroquinoline was resolved by fractional crystallization of its diastereomeric salts with (+)-dibenzoyltartaric acid. Absolute configuration was assigned by x-ray crystallographic analysis. Established chemical procedures allowed preparation of optically pure trans dihydrodiol and arene oxide at the 5,6-position of quinoline. The cytochromes P450 in liver microsomes from 3-methylcholanthrene-treated rats favor formation of the (5R,6S)-oxide enantiomer (3:1) which is hydrated exclusively at C-6 by microsomal epoxide hydrolase to form the trans-(5R,6R)-dihydrodiol. A novel dioxolane route provided optically pure cis dihydrodiol of known configuration from the bromohydrin. *P. putida* (UV4) was then shown to form optically pure (-)-cis-(5R,6S)-dihydrodiol on metabolism of quinoline. In the course of these studies, a prior literature assignment of absolute configuration to 5-hydroxy-5,6,7,8-tetrahydroquinoline was shown to be incorrect.

K-Region arene oxides are prevalent, mutagenic metabolites of polycyclic aromatic hydrocarbons in mammals. These often toxic metabolites are readily inactivated by microsomal epoxide hydrolase. In preparation for kinetic studies of a wide variety of K-region arene oxides with microsomal epoxide hydrolase, we have undertaken detailed studies of the acid-catalyzed solvolysis of the planned enzyme substrates in order to understand the chemical factors underlying the reactivity of these compounds. Solvolysis of benz[a]anthracene 5,6-oxide (BA-O) along with seven methyl and bromine substituted derivatives has been studied in 1:9 dioxane-water (0.1 M $NaClO_4$) and in methanol at 25 °C. These substitutions result in >150-fold differences in their rates of acid-catalyzed solvolysis and cause marked changes in the distribution of solvent adducts and phenols resulting from isomerization. Optically pure BA-O, 7-MBA-O, 12-MBA-O, and 7,12-DMBA-O as well as their optically pure trans dihydrodiols were utilized to determine the point of attack by water in the hydrolysis reactions. For solvolysis in methanol, the distribution of regioisomeric methanol adducts establishes relative rates at the two K-region positions. Rate enhancement on methyl substitution is largest (k_H+, ca. 5-fold) when the methyl group is present in the hindered bay region (C_1 or C_{12}) or adjacent to the epoxide at C_7. The combined effect of two methyl groups (7,12-DMBA-O) is additive (ca. 25-fold). Theoretical calculations (molecular mechanics by PCMODEL-PI and ab initio by GAUSSIAN 86 and 88 programs) of carbocation stability indicate the importance of steric factors in determining relative reactivity and types of products formed from substituted benz[a]anthracene 5,6-oxides. In particular, steric constraints imposed by a methyl group in a bay region or peri to the epoxide result in a conformational inversion of the carbocation becoming a dominant factor in product determination.

The diastereomeric 7,8-diol 9,10-epoxides metabolically derived from benzo[a]pyrene react with the purine bases in nucleic acids to alkylate their exocyclic amino groups. The major adducts formed by polyguanylic acid and the enantiomers of DE-1 (the diastereomer in which the benzylic 7-hydroxyl group and the epoxide oxygen are cis) have been shown to result from cis opening of the epoxide by the N-2 amino group of guanine, rather than trans opening as had been previously reported. Four adducts resulting from alkylation of the exocyclic N-6 amino group of adenosine 5′-monophosphate by racemic DE-1 have been prepared and characterized. In addition, a major adduct formed from adenosine 5′-monophosphate and (-)-(7R,8S)-diol (9R,10S)-epoxide-1, but not from its (+) enantiomer, has been identified as a product of alkylation of the 2′-hydroxyl group of the sugar. The results provide additional support for our previously proposed correlation between the signs of the circular dichroism bands of these adducts and their absolute configurations at the N-substituted benzylic carbon atom.

Hydrolysis reactions of benzo[a]pyrene 7,8-diol-9,10-epoxide isomers DE-1 (noncarcinogen) and DE-2 (carcinogen, benzylic 7-hydroxyl group and the epoxide oxygen are trans) in 1:9 dioxane-water solutions are catalyzed by a series of general acids consisting of $Cl_2CHPO_3H^-$, $ClCH_2PO_3H$-, $H_2PO_4^-$, and $C_2H_5PO_3H^-$. For the hydrolysis of DE-1 catalyzed by H_3O^+, H_2O, and the above series of general acids, a plot of log k_{HA} *vs* pK_a gave a Bronsted α of 0.39. A similar Bronsted plot for the hydrolysis of DE-2 gave an α of 0.40. It is concluded that the mechanism of the hydrolyses of both DE-1 and DE-2 catalyzed by the above general acids with pK_a's <ca. 8, including H_3O^+, must occur by concerted proton transfer and benzyl C-O bond cleavage to yield carbocation intermediates. Dipolar intermediates are ruled out. An intermediate in the spontaneous reaction of DE-1 was trapped, subsequent to its rate-limiting formation, by azide and N-acetylcysteine anions. It is proposed that the rate-limiting step for the spontaneous reaction of DE-1 is formation of a benzylic carbocation intermediate, with a neutral water molecule acting as a proton donor. The rate constant for reaction of this carbocation with solvent is estimated to be $1.7 \times 10^7\ s^{-1}$. Trapping of an intermediate by azide and N-acetylcysteine anions subsequent to a rate-limiting step in the spontaneous hydrolysis of DE-2 was not detected. Thus, a possibly important difference between DE-1 (noncarcinogen) and DE-2 (carcinogen) in neutral water is that biochemical nucleophiles can react with the carbocation of DE-1 subsequent to its rate-limiting formation while DE-2 probably reacts mainly by nucleophilic attack on the neutral epoxide. These divergent mechanisms may account in part for differences in DNA-adduct distributions observed for the two diastereomers.

The restricted ability of deoxyribonuclease I/snake venom phosphodiesterase digestion to liberate deoxyadenosine (dA) nucleotide/bay-region diol epoxide adducts of 7,12-dimethylbenz[a]anthracene from DNA has now been observed with the dA adducts of benz[a]anthracene and benzo[c]phenanthrene diol epoxides. The micrococcal nuclease/spleen phosphodiesterase digestion used in the original ^{32}P-postlabeling procedure developed by Randerath to determine DNA adducts also failed to liberate dA nucleotide adducts quantitatively. Thus either method can potentially lead to an underestimation of the extent to which dA has been modified in DNA. The two digestion procedures exhibit systematic and mostly opposite stereoselectivity in the pattern of dA adducts resistant to digestion, which suggests that these adducts may have had preferred orientations within modified DNA that are determined by whether they have the R or S absolute configuration at C-1 of the hydrocarbons.

SECTION ON OXIDATION MECHANISMS

Mechanistic Enzymology of HIV Proteins.

A spectrophotometric assay for the HIV-1 protease based on the hydrolysis of a chromogenic peptide, Ac-Lys-Ala-Ser-Gln-Asn-Phe(NO_2)-Pro-Val-Val-NH_2 (peptide I), is being used to investigate in detail the mechanism whereby high salt concentrations accelerate peptide bond cleavage by retroviral aspartyl proteases as well as the mammalian aspartyl protease, pepsin. Concentrations of sodium chloride up to 4.5 M accelerated the hydrolysis of Thr-Phe-Gln-Ala-Phe(NO_2)-Pro-Leu-Arg-Glu-Ala (peptide II) catalyzed by the protease of avian myeloblastosis virus (AMV). The effect was exclusively on K_m and resulted in an approximately 125-fold increase in k_{cat}/K_m between 1.0 and 4.5 M salt. Similar effects have been observed by us for the cleavage of peptide I catalyzed by HIV-1 protease, and have also been reported by others for cleavage of several peptide substrates by this enzyme. This salt effect appears to be a general property of aspartyl proteases; i.e., very similar behavior is observed with pepsin. Since pepsin is not a dimeric protein, acceleration of enzymatic catalysis by high ionic strength cannot result simply from an effect on the dimerization of subunits that is required for the activity of retroviral aspartyl proteases. With the AMV protease, high salt concentration has an identical effect on K_m for peptide II and K_i for pepstatin A. Thus, high salt appears to affect predominantly the binding of substrates or inhibitors, and we propose that it may enhance hydrophobic interactions between the substrate or inhibitor and the enzyme's active site.

Constructs of the HIV-1 protease containing flanking Pol region sequences have been expressed as fusion proteins with the maltose-binding protein of the *malE* gene of *Escherichia coli*. After purification by affinity chromatography on cross-linked amylose columns, active 11-KDa protease was obtained by autoprocessing of the fusion protein, which occurred upon denaturation in 5 M urea followed by renaturation. Rapid column purification gave an HIV-1 protease preparation of >95% purity (specific activity ~8500 pmol min^{-1} g protease^{-1}) with an overall yield of about 1 mg/L of culture. Incubation of an inactive mutant protease fusion protein with the purified wild-type protease resulted in specific *trans* cleavage and release of the mutant protease. Mutations that block either the N- or C-terminal protease cleavage sites influence the cleavage at the non-mutated site. These mutated full-length and truncated fusion proteins possess very low levels of protease activity.

In infectious virions, the active form of reverse transcriptase of HIV-1 is a heterodimer consisting of subunits of 66 and 51 kDa. The amino terminal portion of the 66 kDa polypeptide contains the polymerase domain and is separated from the carboxyl terminal portion which contains the RNase-H domain by a central region thought to mediate subunit binding. We have identified two conserved amino-acid repeat motifs within this central region that might be involved in subunit binding: the first is a variation of a "leucine zipper" motif that consists of four leucines and one threonine, and the other is an unusual tryptophan repeat motif. These two amino acid repeat motifs are conserved in essentially all HIV-1 and HIV-2 RT sequences analyzed to date, but are not found in RT sequences of other retroviruses except in that of simian immunodeficiency viruses (SIV) where the tryptophan motif is present.

SECTION ON OXIDATION MECHANISMS

Mass Spectrometry of Drugs, Metabolites, Natural Products and Macromolecules.

Snake Pheromones: A study of the production of sex-related pheromones of female garter snakes has been concluded and it has been found that only those that are mature and capable of reproduction produce the "attractive" pheromone material. We identified these "attractive" pheromones as a series of long chain unsaturated methyl ketones and these are compared to the presence of the normal saturated methyl ketones to establish the "attractiveness". A second effort involved the structural identification of the equivalent compounds in the Brown Tree snake from Guam. This snake is responsible for the total eradication and extinction of three species of birds on Guam. In addition, they are aggressive and bit humans, with children being especially susceptible to the venom. They have already cost the government over 25 million dollars in power outages as well. Methods were developed to locate the two unsaturations in the methyl ketone series present in these snakes.

Biologically Active Molecules from Plants and Marine Organisms: Collaborations on the structural investigation of biologically active natural products from plants and marine organisms has continued. The compounds investigated are those that show promising antiviral, antibacterial and, especially, anti-AIDS potencies, or are synthetic derivatives of these compounds. Compounds with high anti-AIDS activities have been identified.

Natural Benzodiazepines in Brain and Plasma: As part of an investigation into the presence and identity of natural benzodiazepines in body tissues and fluids, very sensitive methods have been developed/improved to measure the level of known benzodiazepines in human plasma in patients. Further work is continuing on the identification and measurement of structurally unknown variants.

Calcium Channel Blocker: Effort has been expended to establish the identity of a natural calcium channel blocker and its final identity is almost confirmed. A compound has been identified and work is continuing to establish its structure.

Adenosine Receptor Agonists and Antagonists

Biochemical and behavioral effects of potent and/or selective adenosine receptor agonists and antagonists continue to be investigated with membranes, cultured cells, organ bath preparations and in vivo with respect to behavior. Further adenosine analogs and heterocycles are being evaluated for selectivity towards A1, A2A and A2B receptors.

BEHAVIORAL EFFECTS OF A_1- AND A_2-SELECTIVE ADENOSINE AGONISTS AND ANTAGONISTS: EVIDENCE FOR SYNERGISM. The locomotor effects in mice of selective A_1 and A_2 adenosine agonists, antagonists, and combinations of agonists were investigated using a computerized activity monitor. The A_2-selective agonist APEC, an amine derivative of CGS21680, was a more potent locomotor depressant than its amide conjugates. The rank order of potency after intraperitoneal injection for adenosine agonists was NECA (ED_{50} 5.8 nmol/kg) > APEC (ED_{50} 25 nmol/kg) > N^6-cyclohexyladenosine (CHA) (ED_{50} 270 nmol/kg). An A_1-selective, centrally acting, adenosine antagonist 8-cyclopentyltheophylline (CPT) (10 mg/kg), completely reversed the locomotor depressant effects of CHA (A_1-selective) and NECA (non-selective) at doses of agonists as high as twice the ED_{50} and shifted the dose response curves to the right, suggesting a primary involvement of A_1 receptors. CPT did not affect the depressant effects of APEC at the ED_{50}, consistent with the A_2-selectivity of APEC. The locomotor effects of APEC and CHA were completely reversed by theophylline, but not by the peripherally active 8-p-sulfophenyltheophylline, indicating central action of the adenosine agonists. The depressant effects of APEC, but not of NECA or CHA, were reversed significantly by an A_2-selective adenosine receptor antagonist, CP-66,713. Low or sub-threshold doses of CHA potentiated the depressant effects of APEC. A sub-threshold dose of CHA did not alter the depressant effect of NECA, while a sub-threshold dose of APEC increased the depressant effects of low doses of NECA. Thus, it appears that A_1 and A_2 selective adenosine agonists have separate central depressant effects, which can be potentiative. The relatively high potency of NECA in vivo could be due to a synergism between central A_1 and A_2 receptor activation by this nonselective agonist.

ACTIVITY OF N^6-SUBSTITUTED 2-CHLOROADENOSINES AT A_1- AND A_2-ADENOSINE RECEPTORS. Radioligand-binding studies of N^6-substituted adenosines at the A_1 and A_2 adenosine receptors of rat brain cortex and rat brain striatum, respectively, show that a 2-chloro substituent does not consistently change the affinity or the selectivity of these analogues for the A_1 receptor. A 2-chloro substituent lowers the characteristic stereoselectivity of the A_1 receptor toward the R diastereomer of N^6-(1-phenyl-2-propyl)adenosine. A 2-chloro substituent consistently increases potency of N^6-substituted adenosines as agonists at an adenosine A_2 receptor stimulatory to adenylate cyclase in PC12 cell membranes.

STRUCTURE ACTIVITY RELATIONSHIPS FOR 2-SUBSTITUTED ADENOSINES AT A_1 AND A_2 ADENOSINE RECEPTORS. A series of fifty 2-alkyloxy-, 2-aryloxy- and 2-aralkyloxyadenosines were screened as inhibitors of binding of [^{3}H]R-phenylisopropyladenosine to A_1-adenosine receptors in rat cerebral cortical membranes and of binding of [^{3}H]N-ethylcarboxamidoadenosine to A_2-adenosine receptors in rat striatal membranes and as agonists at A_2-adenosine receptors coupled to adenylate cyclase in rat pheochromocytoma PC12 cell membranes. The activities are consonant with a hydrophobic binding site in the brain A_2-receptor at a distance from the 2-position of the adenosine ring corresponding to a spacer chain of $-O-CH_2-CH_2-$. There is little lateral steric tolerance at the region of the spacer chain. Interaction with the hydrophobic binding site is greatest in the 2-alkyloxy series for 2-cyclohexylethoxy-, 2-cyclohexylpropoxy- and 2-cyclohexylbutoxy-adenosines and in the 2-aralkoxy series for 2-phenylethoxy-, 4-methyl-phenylethoxy-, 4-chlorophenylethoxy-, and 2-naphthylethoxyadenosine. Structure activity relationships for agonist activity at the A_2-receptor of rat PC12 cells are similar to those for binding at the rat striatal A_2-receptor. Affinities of the 2-substituted adenosines at the rat cerebral cortical A_1-receptors are not as markedly altered by structural changes and are in almost all cases 2 to 100-fold less than the affinity of the 2-substituted adenosine for the rat striatal A_2-receptor. A striking exception is 2-hydroxyethoxyadenosine, which has a 10-fold higher affinity for the A_1 receptor than for the A_2 receptor. There is an excellent correspondance of the present data on rat A_2-receptors with the reported potencies of these 2-substituted adenosines as coronary vasodilators in Langendorf guinea pig heart preparations.

AGONIST ACTIVITY OF 2- AND 5'-SUBSTITUTED ADENOSINE ANALOGS AND N^6-CYCLOALKYL DERIVATIVES AT A_1- AND A_2-ADENOSINE RECEPTORS COUPLED TO ADENYLATE CYCLASE. The activity of N^6-cycloalkyl derivatives of adenosine, 2-chloroadenosine, 5'-chloroadenosine and N-ethylcarboxyamidoadenosine (NECA) and of 2-fluoroadenosine and 5-methylthioadenosines were compared at the A_1-adenosine receptor inhibitory to adenylate cyclase in rat fat cell membranes and at the A_{2A}-adenosine receptors stimulatory to adenylate cyclase in rat PC12 cell membranes. The N^6-cycloalkyl derivatives in all cases were more potent (4 to 23-fold) than the parent compound at the A_1-receptor, and were less potent (1.6 to 11-fold) than the parent compound at the A_2-receptor. The N^6-cyclopentyl-5'-chloroadenosine was the most selective agonist (900-fold) for the A_1-receptor, while 2-fluoroadenosine was the only agonist with some selectivity (2.8-fold) for the A_{2A} receptor. 5'-Methylthioadenosine was a weak agonist at both adenosine receptors. A 2-fluoro derivative of 5'-methylthioadenosine was somewhat more potent.

ANALOGUES OF PYRAZOLOPYRIDINES: EFFECT OF STRUCTURAL ALTERATIONS ON THEIR ANTAGONIST ACTIVITY AT A_1- AND A_2- ADENOSINE RECEPTORS. A series of pyrazolopyridines were evaluated as antagonist of binding of [^{3}H]phenylisopropyladenosine to rat brain A_1-adenosine receptors and inhibitors of [^{3}H]N-ethylcarboxamidoadenosine binding to rat striatal A_2-receptors. Several of the pyrazolopyridines with varying 4-NHR moieties (where R = butyl, aryl, benzyl, phenethyl, cyclopentyl, dimethyl and amino) were found to be as potent or more potent than theophylline at A_1- and A_2- adenosine receptors. None of them exhibited the high potency typical of 8-phenyl-1,3-dialkylxanthines. Most of the analogues showed moderate potencies, which were in the same range seen earlier for pyrazolopyridines tracazolate, cartazolate and etazolate. These later compounds were several fold more potent than theophylline, but were nonspecific for either A_1- or A_2-receptors. In the present investigation one of the analogues, ethyl-4-phenethylamino-1-methyl-1H-pyrazolo[3,4-b]pyridine-5-carboxylate was found to be potent (Ki = 1 μM) and highly specific for A_1 adenosine receptors. It had virtually no activity at A_2-adenosine receptors.

N^6,9-DISUBSTITUTED ADENINES: POTENT, SELECTIVE ANTAGONISTS AT THE A_1-ADENOSINE RECEPTOR. N^6-substituted-9-methyladenines are potent antagonists of the activation of A_1-adenosine receptors. The present study assessed the effect of N^6- and N^9- substituents on the binding of adenines to the A_1- and A_2- receptors, respectively, of rat brain cortex and striatum and also on the antagonism of the A_2-receptor-mediated stimulation of the adenylate cyclase of PC12 cells by NECA. The potency ranking of 9-substituted adenines varied directly with the hydrophobicity of the substituent: cyclopentyl > phenyl > tetrahydrofuryl > ethyl > methyl > 2-hydroxyethyl. The 9-substituted adenines showed little selectivity for either receptor and the R-enantiomer of N^6-(1-phenyl-2-propyl)-9-methyladenine was only 4-fold more potent than the S-enantiomer at the A_1-receptor. An N^6-cyclopentyl substituent increased potency at the A_1-receptor and decreased potency at the A_2-receptor, resulting in selectivity for the A_1-receptor of up to 39-fold. The N^6-cyclopentyl group completely overshadowed the effect of the hydrophobicity of the 9-substituent. A 2-chloro substituent did not alter the potency of an N^6-substituted-9-methyladenine.

CAFFEINE ANALOGS: STRUCTURE-ACTIVITY RELATIONSHIPS AT ADENOSINE RECEPTORS. Caffeine and analogs that contain ethyl, propyl, allyl, propargyl and other substituents in place of methyl at 1-, 3- and 7-positions were antagonists at the two major classes (A_1 and A_2) of adenosine receptors. Potency at both receptors increased as methyls were replaced with larger substituents. Certain analogs with only one of the three methyl groups of caffeine replaced by larger substituents were somewhat selective for A_2-receptors. None of the analogs were particularly selective for A_1-receptors. The presence of polar entities in the substituent at the 1- or 7-position was poorly tolerated at adenosine receptors. Activity of caffeine analogs at A_1- and A_2-adenosine receptors in a variety of systems and cell types have been compared.

A2-ADENOSINE RECEPTORS FROM RAT STRIATUM AND RAT PHEOCHROMOCYTOMA PC12 CELLS: CHARACTERIZATION WITH RADIOLIGAND BINDING AND BY ACTIVATION OF ADENYLATE CYCLASE. Binding assays and assays of activation of adenylate cyclase with the agonists, NECA and CGS21680 in rat striatum and in rat pheochromocytoma cells provide evidence for heterogeneity for A_{2A}-adenosine receptors. The $[^3H]$NECA-binding showed two-components, whereas $[^3H]$CGS21680 bound to one-component in both tissues. The Kd value for the high affinity site labelled with [^{3}H]NECA in PC12 cell membranes (2.3 nM) was lower than that in striatum (6.5 nM). The [^{3}H]CGS21680 binding site showed a Kd value of 6.7 nM and 11.3 nM in PC12 cell and striatum, respectively. A comparison of the ability of a series of agonists and antagonists to inhibit binding of [^{3}H]NECA to A_2 receptors in PC12 cell and striatal membranes indicated that agonists had higher affinities and antagonists had lower affinities in PC12 cell as compared to affinities in striatal membranes. Analysis of activation of adenylate cyclase in PC12 cell membranes indicated that stimulation by NECA involved two components, while CGS21680 stimulated via one component. The Hill coefficient for the NECA curve was markedly less than unity, while that for the CGS21680 curve was closer to unity. In addition, the maximal stimulation by NECA significantly exceeded that caused by CGS21680. In intact PC12 cells, NECA caused a greater accumulation of cyclic AMP than did CGS21680, as had been the case in membranes. In striatal membranes, NECA and CGS21680 showed similar maximal stimulations of adenylate cyclase. Both NECA and CGS21680 were more potent in PC12 cell membranes than in striatal membranes in agreement with binding data. However, in contrast to binding data several antagonists were more potent versus NECA in PC12 cell membranes than in striatal membranes. In toto, the results indicate that A_{2A}-receptors in striatum are similar to, but not identical with the A_{2A}-receptors in PC12 cells. Furthermore, in addition to an A_{2A} receptor, it appears likely that a lower affinity functional receptor, perhaps an A_{2B} receptor, is present in PC12 cells, and this complicates analysis of effects of NECA.

METHYLXANTHINES BLOCK ANTIGEN-INDUCED RESPONSES IN RBL-2H3 CELLS INDEPENDENTLY OF ADENOSINE RECEPTORS OR CYCLIC AMP: EVIDENCE FOR INHIBITION OF ANTIGEN BINDING TO IgE. Activation of a novel adenosine receptor in a rat tumor mast cell line (RBL-2H3 cells) elicits a transient generation of inositol 1,4,5-trisphosphate and an equally transient increase in the level of free cytosol Ca^{2+}. Such responses promote little exocytosis, but markedly enhance the secretory response to antigen. A variety of xanthine adenosine receptor antagonists did not suppress the responses to the adenosine analog 5-N-ethylcarboxamidoadenosine (NECA). However, 3-isobutyl-1-methylxanthine (IBMX) and certain related xanthines inhibited antigen (dinitrophenylated bovine serum albumin, DNP-BSA)-induced generation of inositol phosphates, the increase in level of free cytosolic Ca^{2+} and exocytosis in cells that were primed with a monoclonal DNP-specific IgE. The same compounds inhibited the binding of antigen to cell attached DNP-specific IgE in a highly selective manner. Incorporation of an aromatic or cycloalkyl group in the 8-position of IBMX or theophylline, for example, resulted in compounds that were more potent inhibitors than the parent compounds. Conversely, substituents in the 7- or 9-position of IBMX resulted in inactive compounds. 1,3-Diethylxanthine and 1,3-dipropylxanthine had no activity, suggesting that substituents as large as ethyl or propyl are not tolerated at the 1-position. Inhibition by IBMX was not observed when cells were activated by non-immunological stimulants or when cells were primed with certain other monoclonal preparations of DNP-specific IgE and stimulated by DNP-BSA. Thus, the adenosine receptors in RBL-2H3 cells are distinguishable from A_1- and A_2-receptors by their resistance to xanthines, but some of the xanthines can interfere with antigen-induced responses.

SECTION ON PHARMACODYNAMICS

Interaction between Second Messengers.

Prostaglandin receptors in NIH 3T3 cells

In intact NIH 3T3 murine fibroblasts prostaglandins F2α and E2 (PGF2α and PGE2) induce dose-dependent stimulation of inositol monophosphate generation. PGF2α is > 50-fold more potent than PGE2 in eliciting this response. In streptolysin O-permeabilized NIH 3T3 cells, PGF2α and PGE2 induced dose-dependent accumulations of inositol bis- and tris-phosphates (IP2 and IP3), which were dependent on the presence of a guanine nucleotide (GTPγS 10 μM). Pretreatment of cells for 16 h with 100 nM PGF2α resulted in a significant reduction of not only subsequent PGF2α- and PGE2-induced, but also GTPγS-induced stimulation of IPs formation in permeabilized cells. PGF2α-induced accumulation of IPs was partially inhibited by pretreatment with pertussis toxin (1 μg/ml, 4 h). The inhibition by pertussis toxin was small, and was not related to cAMP formation since forskolin, which activates adenylate cyclase, did not mimic pertussis toxin-induced inhibition. In the same cell line, PGF2α and PGE2 induced a dose-dependent accumulation of cAMP and a dose-dependent potentiation of 0.5 μM forskolin-stimulated cAMP formation. PGF2α and PGE2 were almost equipotent in eliciting both responses. However, PGF2α was less efficacious than PGE2 and in the presence of forskolin, PGF2α at 10 μM induced an inhibitory effect on cAMP accumulation. Such inhibition may be related to PGF2a-mediated phospholipase C activation and subsequent stimulation of protein kinase C, since the phorbol ester PMA, which directly activates protein kinase C, also inhibited forskolin- and PGE2-induced cAMP accumulation. Pretreatment with PGF2α for 16 h did not reduce subsequent stimulation of cAMP accumulation by PGF2α or PGE2. The results indicate that in NIH 3T3 cells two receptors for prostaglandins are present; one that couples to adenylate cyclase probably through Gs and does not exhibit selectivity between PGF2α and PGE2, and a second receptor that couples to phospholipase C through a G protein that is not sensitive to pertussis toxin pretreatment. The latter receptor shows at least 40-fold selectivity towards PGF2α over PGE2. Since long treatment with PGF2α resulted in desensitization of the GTPγS-induced response, it is possible that long exposure to PGF2α may down-regulate the G protein involved in phospholipase C signal transduction.

Effects of Mastoparan and other amphiphilic peptides on phospholipase C in HL-60 cells and cell-free preparations

In differentiated HL-60 cells the amphiphilic peptide mastoparan induces a dose-dependent stimulation of phosphoinositide breakdown with an EC50 value of 9 μM. Such stimulation can be markedly reduced by pretreatment of the cells with pertussis toxin (100 ng/ml, 2 h). In membranes obtained from differentiated HL-60 cells, guanine nucleotides stimulate the formation of IP2 and IP3. Calcium ions also induce phosphoinositide breakdown in this preparation independent of the presence of guanine nucleotides. In HL-60 cell membranes, mastoparan inhibited GTPγS-stimulation of phosphoinositide breakdown with an IC_{50} value of 3 μM. Such inhibitory activity of mastoparan also was present in membranes from cells pretreated with pertussis toxin. Calcium-induced stimulation of phosphoinositide breakdown was not significantly inhibited by mastoparan. The analogs mastoparan-X and polistes mastoparan had similar inhibitory activity, whereas the analog des[Ile]-[Asn]mastoparan was inactive. In permeabilized HL-60 cells mastoparan also inhibited phosphoinositide breakdown. Another amphiphilic peptide, melittin, was inactive in HL-60 intact cells, but similar to mastoparan, inhibited guanine nucleotide-induced phosphoinositide breakdown in HL-60 cell membranes and permeabilized cells. Thus, mastoparan peptides can stimulate phosphoinositide breakdown in intact HL-60 cells, probably through the interaction with a guanine nucleotide binding protein. In permeabilized cells and in cell membranes, mastoparan induces inhibition of guanine nucleotide-mediated phosphoinositide breakdown presumably through an interaction with an intracellular site. The inhibitory action of mastoparan and melittin is probably related to the amphiphilic character of these peptides.

Endothelin receptors are coupled to phospholipase C via G proteins

In permeabilized C6 glioma cells and NIH 3T3 cells, the peptide endothelin 1 (ET-1) in combination with GTPγS stimulates the formation of inositol phosphates. In the presence of 10 μM GTPγS, ET-1 induces the formation of IP2 + IP3 with an EC50 value of 2 nM for C6 glioma cells and 6 nM for NIH 3T3 cells. The analogous peptide ET-3 is less potent than ET-1 in such action. In NIH 3T3 cells, ET-1 + GTPγS-induced formation of IP2 + IP3 could be detected after 1 min of stimulation, and it increased for up to 30 min. ET-1-induced effects were partially reduced by pretreatment of the cells with pertussis toxin (1 μg/ml) in C6 glioma cells, but were unaffected in NIH 3T3 cells. In binding studies in whole C6 cells and NIH 3T3 cells, specific binding for $[^{125}I]$ET-1 was detected. Cross linking of $[^{125}I]$ET-1 in whole C6 cells revealed the presence of two binding proteins for ET-1 of 74 kD and 55 kD. ET-1 at 100 nM inhibited the labelling of both proteins by $[^{125}I]$ET-1. However, ET-3 inhibited the labelling of the 55 kD protein only. The results indicate that in C6 glioma cells and NIH 3T3 cells, receptors for endothelin are present, and are coupled to phospholipase C through guanine nucleotide binding (G) proteins. In C6 cells, endothelin-mediated phospholipase C activation is partially inhibited by pertussis toxin pretreatment. The endothelin receptor involved in phospholipase C stimulation in C6 cells seems to correspond to a 74 kD protein, which binds ET-1 but not ET-3.

SECTION ON BIOCHEMICAL MECHANISMS

Analogues of Thyrotropin-releasing Hormone

In addition to governing the release of thyrotropin and prolactin from the pituitary, TRH (L-pyroglutamyl-L-histidinyl-L-prolineamide) is known to exert a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise in the treatment of circulatory shock and/or CNS ischemic damage, as a promoter of the regeneration of injured spinal cord, as an antidepressant and in the amelioration of symptoms associated with amylotrophic lateral sclerosis (ALS). However, the great variety of its biological effects presents a serious drawback to its use as a drug for specific purposes. Our early studies with synthetic analouges of TRH (involving modification of the imidazole ring of histidine) had suggested that some of the central actions of TRH are not mediated through the recognized high affinity TRH receptors and that appropriate analogues may achieve the long sought specificities of action.

Receptor binding studies with some of our new analogues have made it clear that endocrine and various centrally mediated actions of TRH involve uniquely different mechanisms and that, after several decades of effort in various laboratories, the separation of these activities has been achieved. Thus, 4(5)-NO$_2$-Im-TRH is highly selective for CVS activity, and may be useful in the treatment of various forms of shock without any endocrine effects. On the other hand, Nva^2-TRH is a selective analeptic agent without effect on the cardiovascular system; this analogue has served as a research tool for delineation of those binding sites in rat brain which may mediate the analeptic effects of TRH and its analogues. Computer-assisted structure-activity analysis of a large number of imidazole-substituted analogues has helped us design more selective and potent analogues, as well as photoaffinity labels for TRH receptors. Thus, our predictions of the vital role of imidazole-NH tautomer preference in determining selectivity in binding by brain receptors has been validated by the demonstration that 1-Me-5-NO$_2$-Im-TRH binds ca. 3000 times as tightly as 1-Me-4-NO$_2$-Im-TRH. Recently, we have carried out receptor-binding analysis of various TRH analogues with subtle backbone modifications (replacement of the peptide amide with a thioamide surrogate); these studies have allowed us to identify subtle differences in the high affinity TRH receptors in rat pituitary and brain. Current efforts are devoted to the synthesis and evaluation of analogues of TRH designed to meet the requirements of our new concept of "receptor-activated affinity labels" (RAAL's). Ideally, such a compound would block a receptor site selectivity and indefinitely under conditions of clinical use.

SECTION ON BIOCHEMICAL MECHANISMS

Stereopopulation Control in Drug Delivery and Enzyme Simulation.

Enzymes accelerate organic chemical reactions by 10-20 powers of ten over their closest test-tube counterparts. For example, at pH 7 and ambient temperature, the half time for conversion of adenosine to inosine by adenosine deaminase is 1.9 nsec; in the absence of enzyme, the same reaction would require nearly 15,000 years! In order to account for this remarkable catalytic power of enzymes, it is usually considered that the activation free energy is contributed both by binding of the substrate to the enzyme (step 1) and by chemical transformation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2. We have proposed, however, that the overall catalytic process is more easily justified on the assumption that the first step contributes a more significant share of the activation energy than is generally accepted. To support this theory, we have synthesized a large variety of test-tube models which stimulate the bound substrate by being frozen into a single, very favorable conformation and by having the interacting groups brought into the closet possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions (independently of any functional assistance) at rates comparable to those catalyzed by enzymes, and show that the protein raises both the entropic and ennthalpic components of the substrate by binding it in a single, rigid conformation. Recent work has involved a study of steric and electronic effects on NMR and IR spectra across tight space rather than through covalent bonds. These studies show that kinetic and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limit of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo group. After considerable effort, this goal has been reached and further work is in progress. As part of our studies of practical application of stereopopulation control, we have been exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport from the gut to the circulatory system to the brain by the temporary masking of charge within the molecule, by improvement in lipophilicity and by regeneration based simply on anomalous local pH values in receptor sites. A number of neutral derivatives of dopamine and L-dopa have been synthesized and are undergoing evaluation as prodrugs for penetration of the blood-brain barrier and as slow-release precursors in the blood stream. Protection from premature oxidation of the catechol function and increased lipophilicity have been introduced by the use of the cyclic phenylboronate ester. Model studies show that the boronate the oral effectiveness of polar antibiotics, similar nitroaryl protecting groups are being explored. Several such analogs of penicillin have been synthesized and will be submitted for evaluation.

Chemistry of Bioimidazoles.

Perfluoroalkylimidazoles have attracted considerable interest because of their utility in providing lipophilic sites in medicinal agents and because of their potential use as "receptor-activated affinity labels" (RAAL's) for irreversible blocking of receptors in chemotherapy. The simplest and most general methods for perfluoroalkylation of all heterocyclic rings have been developed in this laboratory and are based on the photochemical generation of perfluoroalkyl radicals. Photochemical perfluoroalkylation of imidazoles leads to mixtures of 2- and 4-isomers while the reaction with pyrroles occurs only at C-2. The practicality of the method for complex substrates has been demonstrated by the facile trifluoromethylation of histidine in peptides (e.g., TRH), followed by HPLC separation of the isomers. Exposure of the trifluoromethyl derivatives of TRH to aqueous ammonia leads to their conversion to the corresponding cyano derivatives. All of these derivatives show very selective binding to low affinity TRH receptor sites in the brain. We have now developed a procedure for regiospecific substitution by blocking all undesired sites with the methylthio group, which is subsequently removed with Raney nickel. Thus, 3-perfluoroalkylpyrroles have been made for the first time. Perfluoroalkylimidazoles have been found to undergo facile nitration and halogenation without destruction; therefore, methods are now available to prepare photosensitive ligands (azido, diazo) and ligands labeled with radioiodine. We have also synthesized pentafluoroethylhistidine and histamine isomers by photochemical radical substitution. These compounds are converted by base into the corresponding trifluoroacetyl derivatives, which have such reactive carbonyl groups that they may serve as affinity labels for bioimidazole-binding sites. Upon treatment with methanolic base, the trifluoromethyl analogs can be converted into trimethoxymethyl and the pentafluoroethyl derivatives into ketals. These ortho functionalities are also of interest as potential covalent affinity labels.

Over the past 16 years, we have found consistently that 2-X-bioimidazoles (especially X = fluoro and iodo) have a broad range of strong biological activities but the corresponding 4-X-bioimidazoles are essentially inactive. We now believe that 2-X-bioimidazoles are recognized and bound to receptors because they have the same NH-tautomer preference as natural bioimidazoles, while the 4-X series prefer the unnatural tautomer. While 4-X-bioimidazoles are readily accessible by direct electrophilic substitution, 2-X-bioimidazoles can be obtained only by indirect and, often, very tortuous routes. Prior to our major efforts in this area, the majority of 2-X-bioimidazoles were totally unknown. The numerous methods available for simple imidazoles are not applicable to bioimidazoles, because the additional functional groups cannot be adequately protected and because chirality should be preserved. Thus, nonclassical methods (e.g., photoradical substitution, electrochemistry, B-12 catalysis, cobalt oxygen carriers, one-electron reduction, singlet oxygen oxidation) have been developed to fit these gaps. The past year has seen the discovery of three major new synthetic methods: (1) the totally specific electrophilic dehalogenation of 2,4-dihaloimidazoles to give 2-haloimidazoles, thus making 2-iodohistidine very easily available for antimalarial studies; (2) the use of transient ylid-carbenes to provide a route to 2-X-imidazoles not otherwise accessible; (3) the discovery that 2-aminoimidazoles are readily oxidized to 2-nitroimidazoles by singlet oxygen, thus providing inexpensive routes to new radiosensitizers and selective cytotoxic agents for cancer chemotherapy.

A number of our 2-X-imidazoles are derived from the corresponding 2-aminoimidazoles. For the past 15 years, we have produced complex 2-aminoimidazoles by slow catalytic hydrogenolysis of 2-arylazoimidazoles. Unfortunately, guanidine resonance in 2-aminoimidazoles is sufficiently strong to effect significant dearomatization of the ring, and the 4,5-double bond now behaves like that of an isolated vinylamine. Thus, the double bond is readily reduced during hydrogenolysis of the azo function, or even with aqueous sodium borohydride. As a result, our hydrogenolysis step has never provided yields of 2-aminoimidazoles greater than 50%. Several years ago, we introduced the use of formamidinesulfinic acid [JOC 49, 1951 (1984)] to achieve the same reduction more efficiently for certain classes of 2-arylazoimidazoles. We have now found that transfer hydrogenation (ammonium formate, Pd-on-charcoal) is a far better technique, cutting reduction time from 7 days to 5 hours and eliminating production of any detectable dihydro products.

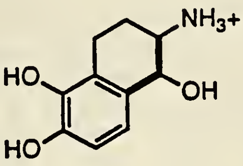
SECTION ON DRUG RECEPTOR INTERACTIONS

Halogenated Biogenic Amines in Biochemistry and Pharmacology.

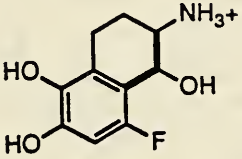
Biological Properties of Fluorinated Amines.

1) Fluorinated Adrenergic Agonists as Probes for Mechanisms of Drug-Receptor Interactions. As reported previously, in every example of phenethanolamine adrenergic agonists we have examined, we have observed that fluorine at the 6-position greatly reduces binding of the agonist at ß-adrenergic receptors, while fluorine in the 2-position has a similar effect at *α*-adrenergic receptors. We also showed that the semi-rigid analogue (1b) of 6-fluoro-NE, a compound that has one structural feature (conformation) that favors ß-adrenergic activity and another structural feature (a "6"-fluoro substituent) that disfavor ß-adrenergic activity, has greatly reduced ß-adrenergic activity relative to the non-fluorinated analogue (1b). We also found that fluorine at the 6-position (2c) of 2-(*t*-butylamino)-1-(3,4-dihydroxyphenoxy)-2-propanol (2a) causes a greatly reduced affinity at ß-adrenergic receptors, while fluorine at the 2-position (2b) significantly increases affinity. The similar response to fluorine substitution by NE (and related type A agonists), 1a, and 2a suggests that fluorine-induced adrenergic selectivities are not caused by

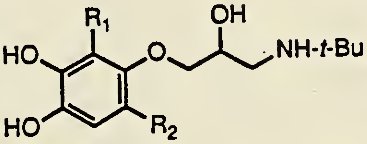
interactions of the fluorine with the side-chain, but more likely are caused by electronic alterations in the aromatic nucleus.



1a

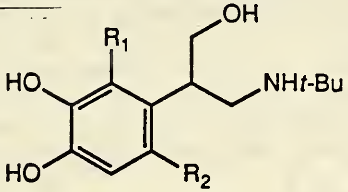


1b



2a: $R_1 = R_2 = H$
2b: $R_1 = F, R_2 = H$
2c: $R_1 = H, R_2 = F$

We also have prepared 2-fluoro (**3b**) and 6-fluoro (**3c**) analogues of the potent β-adrenergic agonist, 3-*tert*-butylamino-2-(3,4-dihydroxyphenyl)-1-propanol (**3a**). While **3a** possesses a side-chain hydroxyl group that could interact with the aromatic fluorine substituent, these interactions (e.g. dipole-dipole repulsions) should be different energetically from those in 2-F- and 6-F-NE. In this series, as in our previous examples, the 6-fluoro substituent markedly reduces β-adrenergic activity while the 2-fluoro substituent has little or no effect on activity. This result provides additional evidence that fluorine substitution somehow changes the ability of the aromatic ring to interact with the adrenergic receptor.



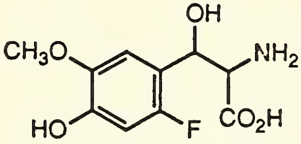
3a: $R_1 = R_2 = H$
3b: $R_1 = F, R_2 = H$
3c: $R_2 = F, R_1 = H$

2) Fluorinated Analogues as ^{18}F-PET Scanning Probes.

a) Synthesis of Metabolites of 6-F-DPOA.

Because of its role as the biological precursor of dopamine, 3,4-dihydroxyphenylalanine (DOPA) labelled with positron-emitting radionuclides has been studied as a potential scanning agent for the in vivo study of dopaminergic neural function. Thus, injected 6-[^{18}F]DOPA crosses the blood-brain barrier, is decarboxylated, and the resulting [^{18}F]-6-fluorodopamine (6-[^{18}F]FDA) is taken up and stored in central dopaminergic neurons. Quantitation of stored 6-[^{18}F]FDA by PET has been used to study central dopaminergic function, both in normal and abnormal subjects.

Successful application of 6-[^{18}F]FDOPA and 6-[^{18}F]FDA (or any positron-emitting agent) as PET-scanning agents requires a firm knowledge of the identity of positron-emitting species present in the experimental subject as a function of time following injection. This presumes a knowledge of the metabolism and biodistribution of the administered agent. For several years, we have been studying the biochemistry and pharmacology of several ring-fluorinated catecholamines and amino acids, including fluorinated analogs of DOPA, DA, NE, and epinephrine (EPI), and the results of these studies have helped validate the use of 6-[^{18}F]fluoro analogues in this series for PET studies. However, we have also shown that the presence of fluorine in the 6-position of compounds downstream metabolically from DOPA can alter significantly such pharmacological behavior as receptor-affinity and metabolic rates. Therefore, while a knowledge of the metabolism of the parent DOPA and dopamine is essential for PET, metabolic studies on the fluorinated analogs themselves also are required, and have been carried out, to place PET-scanning studies on solid footing. During the course of our research, and as a part of our involvement with the development of 6-[^{18}F]FDOPA and 6-[^{18}F]FDA, we have synthesized several products of metabolism of 6-FDOPA and 6-FDA, including 2-fluoro-4-hydroxy-5-methoxyphenylalanine (4), 2-fluoro-4-hydroxy-5-methoxyphenylethanolamine (6-fluoronormetanephrine) (5), 6-fluorohomovanillic acid (6-FHVA) (6), 6-fluoro-4-hydroxy-3-methoxyphenyl glycol (6-FMHPG) (7), 2-fluoro-4-hydroxy-5-methoxymandelic acid (8), and 6-fluoro-3,4-dihydroxyphenylacetic acid (6-FDOPAC) (9).



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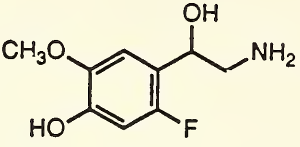

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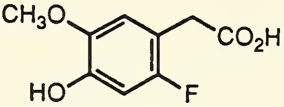

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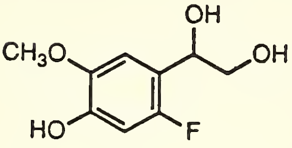

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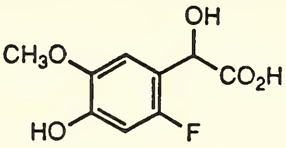

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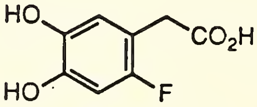

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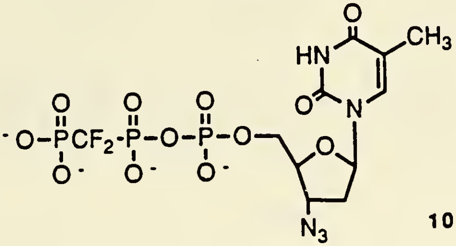
b) $[^{18}F]$-6-Fluorodopamine for PET imaging of Cardiac Sympathetic Innervation. In a related application, 6-$[^{18}F]$FDA is being developed as a PET-scanning agent for the study peripheral adrenergic neural function. FDA does not cross the blood-brain barrier, but is efficiently taken up into peripheral adrenergically innervated organs, such as the heart. The action of dopamine β-hydroxylase converts 6-$[^{18}F]$FDA to 6-$[^{18}F]$fluoronorepinephrine (6-$[^{18}F]$FNE) which, again serving as a false neurotransmitter, is taken up an stored in adrenergic neurons. PET quantitation of 6-$[^{18}F]$FNE then is used to measure the functioning of adrenergic neurons in vivo. The utility of this procedure for assessing cardiac sympathetic nervous function was demonstrated by studying the effects of chemical sympathectomy (6-hydroxydopamine) on PET images after injection of 6-$[^{18}F]$FDA into anesthetized dogs. Whereas clear delineation of the left ventricular myocardium was apparent in the absence of 6-hydroxydopamine, there was very little radioactivity concentrated in the myocardium of sympathectomized dogs. A 10-fold decrease in plasma 6-F-DOPAC in sypathectomized dogs suggests that this metabolite is formed primarily in sympathetic nerve endings (in collaboration with David Goldstein, NHLBI).

3) Development of Potential Chemotherapeutic Agents.

a) Difluorophosphonate Analogue of AZT-5'-triphosphate. The replacement of oxygen in phosphate esters with CF_2 has been an effective strategy in the development of a number of non-hydrolyzable difluoromethylenephosphonate analogues of critical biomolecules. 3'-Azido-3'-deoxythymidine (AZT), used extensively as an approach to the management of human immunodeficiency virus (HIV) infection, is converted in vivo to the 5'-triphosphate (AZTTP), the proximate inhibitor of HIV reverse transcriptase. In the conversion of AZT to its triphosphate, the phosphorylation of AZT-5'-monophosphate (AZTMP) is thought to be the rate limiting step. Based in part on the demonstration of others that β,γ-difluoromethylene-bridged nucleoside triphosphate are often good mimics of parent nucleoside triphosphates, in an initial approach to the design of analogues of AZT that could obviate the requirement for biosynthesis of the triphosphate, we prepared the β,γ-difluoromethylene-bridged analogue (β,γ-CF_2-AZTTP, **10**) which would be expected to be resistant to cleavage at the β,γ linkage. The difluorophosphonate was 30-fold less effective than AZT-triphosphate as a competitive inhibitor of HIV-1 reverse transcriptase.



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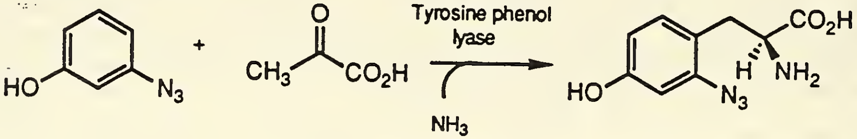
b) 2-Azido-L-tyrosine. As an extension of our research with analogues of biogenic amines and their amino acid precursors, we have initiated synthetic efforts to prepare azido-substituted analogues of such naturally occurring catechols as 3-(3,4-dihydroxyphenyl)alanine (DOPA), dopamine (DA) and norepinephrine (NE). These analogues would have the potential of providing active analogues that, at the same time could function as photoaffinity labels and irreversible inhibitors of key enzymes in the production and metabolism of these neurotransmitters. As a first step in studying the effects of the azido group on biological activity in this series, we have used an enzymatic procedure for the synthesis 2-azidotyrosine (**11**), the biological precursor of DOPA, DA and NE. Preliminary in vitro results suggest that 2-azido-L-tyrosine selectively inhibits the incorporation of tyrosine into protein.



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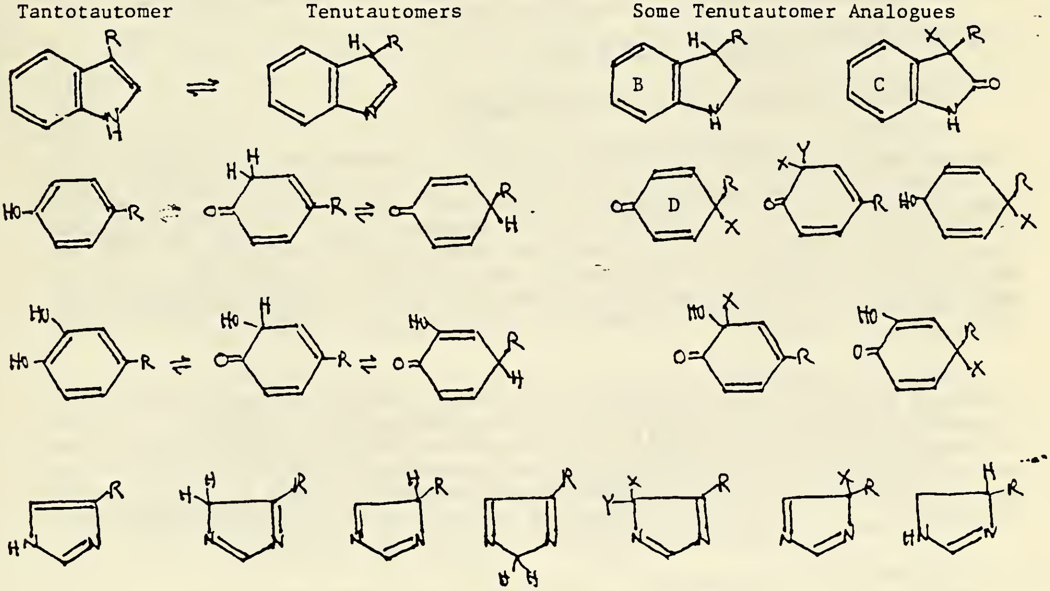
SECTION ON BIOCHEMICAL MECHANISMS

Significance of Ligand Tautomerism in Biorecognition.

A large variety of metabolically important molecules exist as pairs of tautomers, in which the major tautomer (tantotautomer) is so favored energetically over the minor tautomer (tenutautomer) that the latter species cannot even be detected in the equilibrium mixture by any available spectroscopic means. Nevertheless, mechanistic studies have revealed that the tenutautomer is very often the "true" reactant in various chemical transformations. We have proposed that the same phenomenon may exist in biological systems, namely, that the tenutautomers of various metabolites may be the "true" ligands for some enzymes and receptors. This theory provides a new pathway for substrate activation; it also leads to the critical consequence that potential inhibitors and antagonists should be designed as analogues of the tenutautomers, rather than the common practice of mimicking the tantotautomers. Since it is still impossible to examine the detailed structure of a substrate or ligand within a binding site, arguments for the tenutautomer hypothesis must be based on evidence and inference from the behavior of stable analogues of the tenutautomer.

This approach was highly successful and convincing in the case of tryptophan. We found that stable analogues of the indolenine tautomer of L-tryptophan with a tetrahedral carbon at C-3-e.g., 2,3-dihydro-L-trypto-



SCHEME I

phan, oxindolyl-L-alanine and dioxindolyl-L-alanine are potent competitive inhibitors of tryptophan synthase and tryptophanase, enzymes involved in the biosynthesis and degradation of tryptophan. Furthermore, the two enzymes show "mirror-image" specificity, in that the 3R diastereoisomer of each analogue inhibits only tryptophanase while the 3S diastereoisomer inhibits only tryptophan synthase. The diastereoisomers of 3-azido-oxindolyl-L-alanine have also been prepared as potential photoaffinity labels for these enzymes. A logical extension of this series involves the synthesis of tetrahedral analogues of the 5-hydroxy and 5-methoxy derivatives of bioindoles, currently in progress. Additional analogues are being designed and synthesized as potential affinity labels for other bioindole enzymes and receptors.

Some years ago, we discovered a synthetic route to the p-hydroxy-dienone analog of tyrosine by electrolytic oxidation (D,X = OH). Unfortunately, this compound is rather unstable and we are now exploring routes to more stable systems (CN, N_3, OAc, alkyl, etc.). These analogues will be tested as inhibitors of tyrosine phenol-lyase. Even more important tests and applications of the principle involve the synthesis and evaluation of dienone analogues of catecholamines and dihydroxyphenyl-alanine; synthesis of such analogues presents a novel and unprecedented challenge. Studies have also been initiated to explore the synthesis of CH-tautomers of histidine and histamine, for which we have already established a role in test-tube reactions and which we believe to be the "true" substrates in certain enzymatic processes.

SECTION ON DRUG RECEPTOR INTERACTIONS

Functionalized Congeners of Bioactive Compounds.

By the functionalized congener approach to drug design, new analogs are synthesized with the regiospecific inclusion of a functionalized chain at a point which can accommodate molecular modification and a certain degree of steric bulk. The resulting functionalized drug congener may then be attached through an amine or other reactive group on the chain to various organic moieties, such as amines and peptides. The receptor-binding affinity of these analogs is often greater than that of the parent drug and does not necessarily diminish as the molecular weight is systematically increased.

Applications of the functionalized congener approach include the synthesis of probes for receptor studies (for example: radioligands, fluorescent probes, or chemically reactive affinity labels) and the design of drug delivery systems (including targeting and altering the characteristics to allow for passage through membranes). Drug conjugates may be designed in a stepwise approach to optimize certain pharmacological properties, such as potency, specificity, and duration of action.

XAC (xanthine amine congener), a high affinity adenosine antagonist, was designed by this approach. The intrinsic affinity of 8-phenylxanthine analogs, such as XAC, at striatal A2-adenosine receptors is highly species dependent. [^{3}H]XAC, although A1-selective in the rat brain, binds to A2 receptors in rabbit striatal membranes with sufficiently high affinity to serve as a radioligand. In the presence of 50 nM CPX (8-cyclopentyl-1,3-dipropylxanthine), an A1-selective antagonist added to eliminate binding to A1 receptors, [^{3}H]XAC exhibits saturable, specific binding (70% of total) to A2 sites with a Kd of 3.8 nM and a Bmax of 1.23 pmol/mg protein. At 24°C, the association and dissociation rate constants were 0.13 min-1.nM-1 and 0.36 min-1, respectively. Binding was performed for 1 hr, with non-specific binding defined in the presence of 100μM NECA (N-ethylcarboxamidoadenosine). The potency order for antagonists against 1 nM [3H]XAC at rabbit A2-receptors was XAC ≈ Nα-Me-XAC >> CPX = XCC > 1,3-dipropyl-8-p-sulfophenylxanthine > PSPT. The relative potency order for agonists was CGS21680 ≈ NECA > APEC (= 2-(aminoethylaminocarbonylethyl-phenyl-ethylamino)-NECA) > PAPA-APEC > ADAC > R-PIA (N6-phenylisopropyl-adenosine) > S-PIA.

XAC was also used for the isolation of adenosine receptors by affinity chromatography. A$_1$ adenosine receptors (A$_1$AR) acting via the inhibitory guanine nucleotide binding protein inhibit adenylate cyclase activity in brain, cardiac, and adipose tissue. We now report the purification of the A$_1$AR from bovine cerebral cortex. This A$_1$AR is distinct from other A$_1$ARs in that it displays an agonist potency series of N^6-R-phenylisopropyl-adenosine (R-PIA) > N^6-S-phenylisopropyladenosine > (S-PIA) > 5′-N-ethylcarboxamidoadenosine (NECA) compared to the traditional potency series of R-PIA > NECA > S-PIA.

The A$_1$AR was solubilized in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and then purified by chromatography on an antagonist [xanthine amine congener (XAC)]-coupled Affi-Gel 10 followed by hydroxylapatite chromatography. Following purification, sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed a single protein of M$_r$ 36,000 by silver staining, Na^{125}I iodination with chloramine T and photoaffinity labeling with [^{125}I]8-[4-[[[[2-(4-aminophenyl acetyl-amino)ethyl]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine. This single protein displayed all the characteristics of the A$_1$AR, including binding an antagonist radioligand ([^{3}H]XAC) with high affinity (K_d = 0.7 nM) and in a saturable manner (B_{max} > 4500 pmol/mg). Agonist competition curves demonstrated the expected bovine brain A$_1$AR pharmacology: R-PIA > S-PIA > NECA. The overall yield from soluble preparation was 7%.

The glycoprotein nature of the purified A$_1$AR was determined with endo- and exoglycosidases. Deglycosylation with endoglycosidase F increased the mobility of the A$_1$AR from M$_r$ 36,000 to M$_r$ 32,000 in a single step. The A$_1$AR was sensitive to neuraminidase but resistant to α-mannosidase, suggesting the single carbohydrate chain was of the complex type. This makes the bovine brain A$_1$AR similar to rat brain and fat A$_1$AR, but it retains its unique agonist potency series observed in membranes.

Functionalized congeners are also being explored for therapeutic goals. Most clinically available cholinergic drugs (e.g. the agonist pilocarpine and the antagonist atropine) are non-selective in their interaction with muscarinic receptor subtypes. The recent cloning, sequencing, and expression of five separate genes for muscarinic receptors has raised the possibility of developing novel organic compounds that act as agonists or antagonists at one of these subtypes. Selective compounds could be therapeutically useful in treating a variety of diseases, including Alzheimer's disease, cardiac disease, neurogenic bladder, and certain sleep disorders. Furthermore, such specific compounds, by virtue of their subtype selectivity, should be devoid of many of the side effects of currently used compounds.

Alzheimer's disease is associated with a premature decrease in the density of receptors for a number of neurotransmitters in the brain. In particular, a profound degeneration of presynaptic central muscarinic pathways in the cholinergic basal forebrain system has been observed. These pathways originate in the nucleus basalis and in other regions. The postsynaptic muscarinic receptors are though to be mainly intact and amenable to pharmacological manipulation. Therefore, many attempts have been made at treating Alzheimer's patients with cholinergic compounds, with mixed success. One reason for difficulties with the cholinergic approach to treatment of Alzheimer's disease may be that most muscarinic agonists stimulate both pre- and postsynaptic receptors. Agonist action at presynaptic muscarinic receptors may be counter-productive since it inhibits the release of acetylcholine in the remaining fibers. It has been suggested that muscarinic agents like BM5, which are presynaptic antagonists and postsynaptic agonists, are potentially useful in treating Alzheimer's dementia.

In view of the above, a goal is to develop novel and selective muscarinic agonists and antagonists. We have used a functionalized congener approach in the design of new muscarinic ligands. A series of substituted azacycloalkyl analogues of the muscarinic agonist UH5 (N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]acetamide, 1a) were synthesized and evaluated pharmacologically. These compounds were developed as intermediates for further derivatization leading to functionalized congeners of 1a. The compounds were synthesized using a Mannich-type condensation of N-acetyl-N-methyl-propargylamine to various substituted saturated aza-heterocycles. The compounds were screened at a single concentration in competitive binding assays in rat cerebral cortical membranes against either $[^3H]$N-methylscopolamine (at 100 μM) or $[^3H]$oxotremorine-M (at 1 μM) labels. Candidates were then selected for further evaluation of their effect on phosphoinositide (PI) turnover in membranes from A9L cells transfected with cDNA of either m1-muscarinic cholinergic receptors (m1AChRs) or m3AChRs. The analogues were also tested for the inhibition of adenylate cyclase in NG108-15 cells expressing m4AChRs. The azetidine analogue of 1a had a Ki value of 12 nM for the inhibition of [3H]oxotremorine-M binding in rat brain and had an agonist potency at m1-, m3-, and m4AChRs comparable to 1a. The substituted five-

and six-member ring analogues generally had lower binding affinities and were less potent than 1a in stimulating PI turnover. Several compounds were moderately effective in inhibiting cyclic AMP production in NG108-15 cells.

SECTION ON DRUG RECEPTOR INTERACTIONS

Prosthetic Groups for Radiolabeling of Functionalized Drugs and Peptides

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabelled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as ^{18}F, ^{123}I, and ^{125}I, to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor. These prosthetic groups contain amino or carboxylic groups which are to be coupled covalently to functionalized drugs to give conjugates of high affinity at a particular receptor.

Positron emission tomography (PET) based on short-lived isotopes such as 18F and 11C has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of ^{18}F requires rapid and efficient reaction and purification, since the half-life is only 110 minutes. Compounds, such as fluorodeoxyglucose, containing this isotope have been used extensively as an _in_ _vivo_ tracers. ^{18}F positron emission tomography (PET) has been particularly effective in the study of brain metabolism and in scanning receptors and sites of drug uptake. PET scanning allows more than spatial imaging; it provides a look at the biochemical state of an organ.

An objective of our chemical investigation is to provide compounds and a general methodology for rapid introduction of radioactive fluorine into peptides and functionalized drugs. This general methodology for introduction of 18F is hoped to lead to improvements in diagnostic nuclear medicine, and to provide improved brain imaging by positron emission tomography. The reactions through which 18F is introduced into a molecule include nucleophilic displacement of alkyl triflates, aromatic nucleophilic attack, and halogenation of phenols, olefins, and metallo-organics. Benzylic electrophilic centers are subject to attack by active nucleophiles, including "naked" fluoride ion in nonaqueous media.

The feasibility of attachment of the bromomethylbenzoyl prosthetic group in biological systems has been demonstrated. In addition to the many peptides which contain non-essential amino groups, some drug molecules may also be coupled to this prosthetic group without loss of receptor binding.

^{18}F-insulin of high specific radioactivity was synthesized using a new, general methodology for the incorporation of a positron emitter into peptides and proteins. ^{18}F-Insulin is chemically stable and retains the essential biological properties of insulin, as measured in vitro by receptor binding, by stimulation of glucose metabolism in rat adipocytes, and in localization studies in vivo in primates. The synthesis uses 4-bromomethylbenzoyl- ("BMB") as an amide-linked prosthetic group. Nucleophilic displacement of the bromide by cyclotron-produced ^{18}F-fluoride ion forms a 4-fluoromethylbenzamide, 9. There are three free amino groups present on native porcine insulin (A1, B1, and B29), 1. In order to achieve site-specific reaction at the site that is least sensitive in receptor binding (the B1 phenylalanine residue), it was necessary to protect the A1 and B29 amino residues as the t-butyloxycarbonyl (Boc) amines (eg. compound 2) during the synthesis. Since insulin and its derivatives did not withstand the conditions of the fluoride exchange reaction (tetraalkylammonium hydroxide in acetonitrile at 70°C for 10 min), it was necessary to preactivate the B1 site for linking to an intermediate already containing ^{18}F. This was accomplished by treating the HPLC-purified A1,B29-di-Boc-insulin with a large excess of disuccinimidyl suberate (DSS) in DMSO in the presence of triethylamine to give a single major product, A1, B29-di-Boc-B1-succinimidylsuberoyl-insulin, 3. In the next step, an HPLC purified intermediate containing the ^{18}F-FMB group was coupled to the protected and preactivated insulin derivative, to give compound 4, which was deprotected to give the biologically active ^{18}F-insulin derivative, 5. The overall process of reaction, purification, and deprotection was carried out within two half lives (t1/2 = 110 min) to yield a product of sufficient purity to permit in vivo studies. Specific activities of 12-14 Ci/μmole at the time of injection typically were obtained. In in vitro studies, ^{18}F-insulin, studied over the concentration range of 3 pM to 1.5 nM, bound to insulin receptors on human lymphoblastoid (IM-9) cells with an affinity that was equal to or better than that observed for the binding of ^{125}I-insulin. ^{18}F-Insulin that had undergone radioactive decay competed for ^{125}I-insulin binding to insulin receptors in a manner indistinguishable from unlabeled insulin. Further, unlabeled insulin competed with ^{18}F-insulin in a fashion similar to its competition for ^{125}I-insulin, providing further support for the close resemblance of the fluoro- derivative to the iodo- as well as the native peptide. In in vivo studies, Rhesus monkeys were injected with 0.3-1.4 mCi of ^{18}F-insulin and scanned for 30-60 min. Significant uptake of the radiotracer was detected immediately post-injection in liver and kidneys, with delayed uptake in bile and bladder. In the presence of coinjected, unlabeled insulin labeling of the liver decreased by 50%, indicating specific binding of the radiotracer to insulin receptors. Tracer distribution within the first 5 minutes after injection (during which period the tracer was stable in vivo) suggested different kinetics for uptake in liver and kidneys.

Chemical affinity labeling of biopolymers depends on a spontaneous covalent bond formation between a reactive ligand and a specific nucleophile on the receptor protein. Functionalized congeners derived from XAC (an amine congener of 1,3-dipropyl-8-phenylxanthine), a potent A1 selective adenosine antagonist, were derivatized to contain chemically reactive groups capable of reaction with nucleophiles such as amines and thiols on biopolymers.

The 1,3-phenylene diisothiocyanate conjugate of XAC has been characterized as an irreversible inhibitor of A1-adenosine receptors. To further extend this work, a series of analogues were prepared containing a third substituent in the phenylisothiocyanate ring, incorporated to modify the physio-chemical or spectroscopic properties of the conjugate. Symmetrical trifunctional cross-linking reagents bearing two isothiocyanate groups were prepared as general intermediates for crosslinking functionalized congeners and receptors. Xanthine isothiocyanate derivatives containing hydrophilic, fluorescent, or reactive substituents, linked via an amide, thiourea, or methylene group in the 5-position, were synthesized and found to be irreversible inhibitors of A1-adenosine receptors. The effects of the 5-substituent on water solubility and on the A1/A2 selectivity ratios derived from binding assays in rat brain membranes were examined. Inhibition of binding of $[^3H]$N6-phenylisopropyladenosine and $[^3H]$CGS21680 (2-[[4-(2-carboxyethyl)phenylethyl]amino]adenosine-5′-N-ethylcarboxamide) at central A1 and A2-adenosine receptors, respectively, was measured. A conjugate of XAC and 1,3,5-triisothiocyanatobenzene was 894-fold selective for A1-receptors. Reporter groups, such as fluorescent dyes and a spin label, were included as chain substituents in the irreversibly binding analogues, which were designed for spectroscopic assays, histochemical characterization, and biochemical characterization of the receptor protein.

SECTION ON DRUG RECEPTOR INTERACTIONS

Development of Drugs Acting at Adenosine Receptors

Adenosine acts as a neuromodulator in the circulatory, endocrine, immune and central nervous system. The biological activity is associated with two receptor subtypes: the A1-adenosine receptor mediates cardiac and central depressant and antilipolytic activities and is coupled to adenylate cyclase in an inhibitory manner; the A2-receptor is involved in vasodilation and antithrombotic functions, possibly through stimulation of adenylate cyclase. The major class of antagonists, the alkylxanthines, generally acts at both receptor subtypes; there is currently a search for analogs which have high potency, high receptor subtype selectivity, and increased water solubility.

The functionalized congener approach has provided a wide range of potent, selective, and novel analogs at A1- and A2-adenosine receptors. Functionalized congeners of adenosine, in which the derivatization site was the purine N6- or C2-position, were found to be general A1- and A2-selective probes, respectively. Alkylxanthines (the largest class of adenosine antagonists) likewise have been derivatized through the p-position of an 8-phenyl substituent. Potency was defined initially by affinities for adenosine receptors in binding assays and in receptor-mediated effects on adenylate cyclase activity. Selected compounds have been screened in a variety of biological systems, including effects on the cardiovascular system and on behavior.

For both adenosine agonists and antagonists, the presence of a distal amino group enhanced potency. For example, the analogue of XAC in which the primary amino group was replaced by a hydroxy function was 8.5-fold less potent than XAC in the displacement of $[^3H]$R-phenylisopropyladenosine at A1-adenosine receptors. The purine amine congeners (XAC, ADAC, and APEC) served as synthetic intermediates for the synthesis of probes for photoaffinity labeling, chemical affinity labeling, and affinity chromatography of adenosine receptors.

XAC (xanthine amine congener, antagonist), ADAC (adenosine amine congener, A1-agonist), and APEC (2-[(2-aminoethylamino)carbonylethylphenyl-ethylamino]-5'-N-ethylcarbox-amidoadenosine, A2-agonist) were designed as functionalized congeners: i.e., they contain a reactive functional group (an amine) that can be coupled covalently to other molecules resulting in

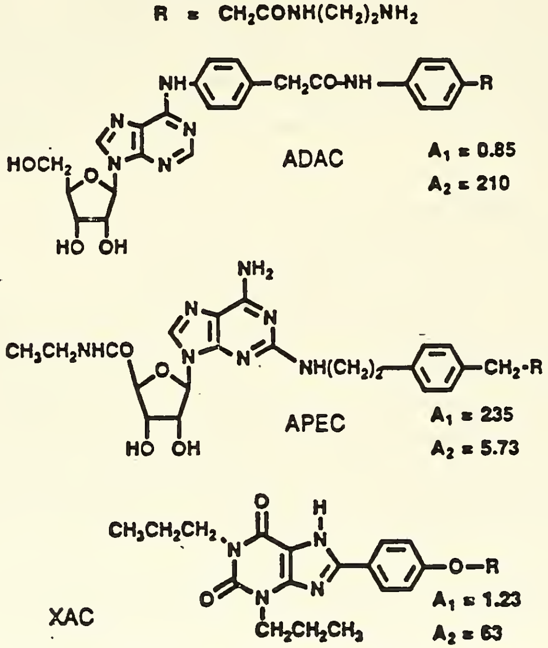


Fig. Purine functionalized congeners (K_i values in nM, versus $[^3H]N^6$-phenylisopropyladenosine at A_1-receptors or $[^3H]$ 5'-N-Et-carboxamidoadenosine at A_2-receptors in rat brain membranes).

further biologically active conjugates. XAC and ADAC are roughly 50-fold and 250-fold selective, respectively, for rat brain A1-adenosine receptors, and APEC is roughly 40-fold selective for rat brain A2-adenosine receptors. Tritiated XAC has been used to analyze agonist and antagonist interactions at A1 adenosine receptors. Conjugates of XAC, ADAC, and APEC have been prepared that contain prosthetic groups for the incorporation of radioisotopes, or contain fluorescent groups for spectrophotometric detection. Such fluorescent groups include fluorescein, rhodamine, and nitrobenzoxadiazole. Purine functionalized congeners may be coupled to agarose affinity matrices or to bifunctional cross-linking reagents for irreversible receptor binding. Conjugates containing biotin to probe receptor topography, fatty acids to utilize lipid delivery, prodrugs, and binary drugs have been developed.

Previous work using sucrose gradient centrifugation and the antagonist radioligand [^{3}H]xanthine amine congener led to the proposal that A_1 adenosine receptors are coupled to a GTP-binding protein (G protein) in the absence of an agonist and that adenosine receptor antagonists bind to free uncoupled receptors with high affinity and coupled receptors with low affinity and cause a destabilization of receptor-G protein complexes. Because agonists form high affinity ternary complexes composed of the agonist, receptor, and G protein, this hypothesis would imply that interactions between adenosine receptor agonists and antagonists, while competitive, would appear to be "noncompetitive" in nature. Interactions between unlabeled and radiolabeled A_1 receptor agonist and antagonist ligands have been investigated using bovine cerebral cortical membranes to further probe this point. The availability of both ^{3}H- and ^{125}I-radioligands allowed us to use both single- and dual-isotope experimental designs. Radioligand antagonist-agonist competition curves along with saturation analyses using filtration and centrifugation to isolate bound radioligand suggested that agonists bind to two sites or receptor states with high affinity and to one site with low affinity. Agonist radioligand saturation curves with or without unlabeled antagonist suggested that antagonists do not bind to all states of the receptor with equal affinity. The computer program EQUIL was used to define models capable of simultaneously fitting all parts of complex experiments in which ^{125}I-N^6-aminobenzyladenosine saturation isotherms with or without 8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX were performed. The data were not compatible with two-independent site models or with ternary complex models involving one receptor and one G protein. The data were fit by a model involving one receptor and two G proteins and by a model involving two receptors and one G protein. Both models suggest that 1) a high percentage of the receptor(s) is coupled to a G protein in the absence of an agonist and 2) agonists stablize whereas antagonists destabilize precoupled receptor-G protein complexes. Because of this, competitive interactions between A_1 agonists and antagonists appear noncompetitive in nature.

Mammalian A_2-adenosine receptor binding subunits (A_2AR) can be visualized by covalent labeling with the photoaffinity crossliking ligand

^{125}I-PAPA-APEC or directly with the azide derivative described in this paper. The protein comprising the A_2-adenosine receptor binding subunit migrates with a M_r of 45,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In this study, the glycoproteins representing the radiolabeled A_1- and A_2adenosine receptor binding subunit from bovine brain were compared by partial peptide maps and following treatment with exo- and endoglycosidases. Peptide maps using two separate proteases reveal that the A_1- and A_2-adenosine receptor binding subunits share no common peptide fragments by two-dimensional gel electrophoresis. Endoglycosidase F treatment of labeled A_2AR results in a single labeled peptide of M_r 38,000 without intermediate peptides, suggesting a single N-linked carbohydrate chain. The labeled A_2AR demonstrates a sensitivity to neuraminidase, as evidenced by an increased mobility on gel electrophoresis, suggesting the receptors contain a glycan component containing terminal sialic acid. Treatment of the labeled A_2AR with α-mannosidase reveals two distinct populations of A_2ARs, one of which is sensitive and the other resistant to the enzyme. The nonadditivity of sequential treatments with the two exoglycosidases suggests, a heterogeneous population of A_2AR containing either complex- or high mannose-type carbohydrate chains. These data suggest the A_2AR is a M_r 45,000 glycoprotein with a single carbohydrate chain of either the complex or high mannose type. In addition, the A_1- and A_2ARs are distinct glycoproteins, as evidenced by their differing molecular weights (before and after deglycosylation) and distinct peptide maps.

Agonist binding to the A_2 adenosine receptor (A_2AR) and its regulation by guanine nucleotides was studied using the newly developed radioligand ^{125}I-PAPA-APEC and its photoaffinity analog ^{125}I-azido-PAPA-APEC. A single protein of M_r 45,000, displaying the appropriate A_2AR pharmacology, is labeled in membranes from bovine striatum, PC12 cells, and frog erythrocytes. In DDT_1MF2 cells the labeled protein has a slightly lower molecular weight. Incorporation of ^{125}I-azido-PAPA-APEC into membranes from rabbit striatum, however, reveals two specifically labeled peptides (M_r ~47,000 and 38,000), both of which display A_2AR pharmacology. Inhibition of protease activity leads to a decrease in the amount of the M_r 38,000 protein, with only the M_r 47,000 protein remaining. This suggests that the M_r 38,000 peptide is a proteolytic product of the M_r 47,000 A_rAR protein. In membranes containing the intact undigested A_2AR protein, guanine nucleotides induce a small to insignificant decrease in agonist binding, which is atypical of stimulatory G_s-coupled receptors. This minimal effect is observed in rabbit striatal membranes prepared in the presence of protease inhibitors, as well as in the other tissues studied. Binding to rabbit striatal membranes that possess the partially digested receptor protein, however, reveals a 50% reduction in maximal specific agonist binding upon addition of guanine nucleotides. Inhibition of proteolysis in rabbit striatum, on the other hand, results in a diminshed ability of guanine nucleotides to regulate agonist binding. Thus, the enhanced effectiveness of guanine nucleotides in rabbit striatal membranes is asociated with the generation of the M_r 38,000 peptide fragment. Guanosine 5′-(β,γ -imido)triphosphate reduces photoaffinity labeling by 55% in the M_r

38,000 protein, whereas the labeling is decreased by only 28% in the M_r 47,000 receptor protein.

Our data, therefore, suggest that, unless proteolysis occurs, the A_2AR in all tissues studied is tightly associated with the G_s protein and displays minimal guanine nucleotide modulation of agonist binding, which makes the A_2AR an atypical stimulatory receptor.

Previously we have shown that the improvement of cold tolerance by theophylline is due to antagonism at adenosine receptors rather than inhibition of phosphodiesterase. Since theophylline is a nonselective adenosine receptor antagonist for both A_1 and A_2 receptors, the present study investigated the adenosine receptor subtype involved in theophylline's action. Acute systemic injection of selective A_1 receptor antagonists (1,3-dialkyl-8-aryl or 1,3-dialkyl-8-cyclopentyl xanthine derivatives) significantly increased both the total and maximal heat production as well as cold tolerance. In contrast, injection of a relatively selective A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine failed to significantly alter the thermogenic response of the rat under cold exposure. Further, the relative effectiveness of these compounds in increasing total thermogenesis was positively correlated with their potency in blocking the A_1 adenosine receptor ($r = .52$, $p < 0.01$), but not in A_2 adenosine receptor ($r = .20$, $p < 0.2$). It is likely that the thermally beneficial effects of adenosine A_1 antagonists are due to their attenuation of the inhibitory effects of endogenously released on lipolysis and glucose utilization, resulting in increased substrate mobilization and utilization for enhanced thermogenesis.

One of the major side effects of the methylxanthines and adenosine receptor antagonists caffeine and theophylline is the provocation of seizures. With the availability of highly selective adenosine receptor antagonists it was of interest to investigate if they would also induce seizures. In experiments with mice it was found that also highly selective adenosine receptor antagonists like Xanthine Amine Congener are able to induce seizures. The therapeutic usefulness of highly selective adenosine receptor antagonists as e.g. cognitive enhancers in dementia syndromes is thus limited by this serious side effect.

SECTION ON BIOCHEMICAL MECHANISMS

Aldose Reductase Inhibitors

Efforts to retard and ameliorate late-onset diabetic complications have been directed toward reversible and/or irreversible inhibition of the enzyme, aldose reductase. These complications affect the eye (cataracts), kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of abnormally high intracellular concentrations of sorbitol, in turn resulting from the reduction of excess glucose symptomatic of diabetes. Aldose reductase is the enzyme responsible for sorbitol formation.

Our studies on the synthesis of inhibitors of tryptophan-metabolizing enzymes have produced oxindole derivatives which are somewhat analogous in overall geometry to certain commercial compounds which had reached advanced clinical trials as aldose reductase inhibitors. In vitro assays have revealed that, while these oxindole derivatives (lactones) are not significantly active, their hydrolysis products are as potent as any others thus far developed. Although these chiral compounds are not amino acids, we have succeeded in the use of chymotrypsin to achieve their resolution by selective ester hydrolysis. Assignment of stereochemistry has been achieved through x-ray crystallography and through the novel approach of destroying the known chiral center while retaining the unassigned center in a diastereoisomer. The introduction of certain substituents on the benzene ring of the oxindole has been found to increase inhibitor activity several fold. Additional analogues have been prepared which are expected to serve as irreversible affinity and photoaffinity labels for the enzyme. Current efforts are being devoted to improvements in yield, in resolution and in enhancement in lipophilicity, with the goal of achieving more effective penetration and transport to the sites of action in vivo. Still another new direction involves the replacement of oxygen by sulfur in the oxindole portion, in order to encourage the formation of a trigonal carbon at C-3. Thus, we hope to learn more about structure-activity requirements. Since our inhibitor series is significantly different in structure and in functional groups from those which had reached clinical trials, we have strong hope that this series will not elicit the serious side effects which ultimately had caused many of the commercial candidates to be abandoned.

SECTION ON BIOCHEMICAL MECHANISMS

Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides.

Introduction of conformational constraints in peptides is a very useful means to probe peptide-receptor interactions and to enhance their potencies and/or selectivity. While local conformational restraints can be imposed by incorporation of unsaturated or small ring amino acids, side chain-side chain cyclization to form cyclic analogues ahs been found to be one of the most useful approaches for the introduction of more general conformational constraints: e.g., a disulfide bond between Cys or Pen residues, or an amide bond between side chains of amino and carboxy trifunctional amino acids. The 13-membered cyclic peptide, H-Tyr-D-Orn-Phe-Asp-NH_2 (cyc-Orn-Asp), is a potent μ-selective opioid peptide. Molecular modeling of this peptide has revealed considerable flexibility in the ring structure, making it difficult to identify a single conformation which may be biologically relevant. In order to restrict further the conformational mobility in such peptides, we have designed and synthesized a novel trifunctional amino acid based on pyrrolidine (PDA). This amino acid, if used in place of Orn or Lys for side-chain-side chain cyclization, is expected to reduce considerably the conformational flexibility in the macrocyclic ring of these peptides owing to the introduction of a bicyclic structure. Moreover, the additional asymmetric center in the pyrrolidine ring may introduce differential stereochemical constraints to the binding of diastereoisomeric peptides, derived from the corresponding diastereoisomeric PDA's. Thus, these peptides may act as probes for delineating the stereochemical topology of the receptor in the vicinity of the ring structure and may have useful biological properties and applications.

Optically active amino acids are being used increasingly as synthons for the preparation of a varity of chiral compounds. PDA's can be expected to be excellent synthons for compounds such as pyrrolizidines with the sterochemistry already defined at two centers. Furthermore, these amino acids may possess antimicrobial or other antimetabolic activities. An efficient method for the cynthesis of all four optically active stereoisomers of PDA has been developed. These amino acids will be used for the preparation of novel bicyclic opioid peptide analogues.

SECTION ON BIOCHEMICAL MECHANISMS

Fluorinated Analogues of Bioactive Peptides

Fluorinated analogues of a wide range of structurally different classes of organic compounds have been prepared and many of these have proven to be important pharmacological and medicinal agents. The advantages of fluorine substitution arise from the small van der Waals radius of fluorine (1.35 A). As a result, fluorine bonded to a trigonal carbon is used effectively as a C-H replacement. Furthermore, because of similar aliphatic C-F and C-O bond lengths (1.39 and 1.43 A, respectively) and because fluorine has some ability to participate in hydrogen bonding, the halogen bonded to a tetrahedral carbon has been used frequently as an OH surrogate, particularly in carbohydrate research and in nuceloside analogues. These steric similarities allow fluorinated compounds to mimic their nonfluorinated parents with respect to recognition by various biological macromolecular systems such as enzymes, transport proteins and receptors. On the other hand, the high electronegativity of fluorine (4.0) can drastically alter electron density distribution in the molecule which, in turn, affects pK values in neighboring functional groups and molecular dipole moments. All these altered physicochemical properties of the molecule, as a result of fluorine substitution, can result in drastically modified biological properties such as potency, transport and receptor selectivity. Furthermore, recent advances in 19-F NMR and electron energy-loss spectroscopic techniques, and 18-F position-emission tomography (18-F PET), have increased the significance and application of fluorinated molecules as biological markers and diagnostic agents.

Although many methods have been developed for fast and efficient incorporation of 18-F and 19-F into various compounds of medicinal importance, such methods have not been generally practical for incorporation into peptides. We have now developed a method for fast, efficient introduction of fluorine regiospecifically into the phenolic ring of tyrosine-containing peptides by use of the electrophilic fluorinating agent, acetyl hypofluorite (AcOF). We have used this method for fluorinating μ-selective opioid peptides. In vitro bioassays and receptor-binding assays have revealed that these fluoro peptides retain their high receptor selectivity. Thus, we have been able to develop a very μ-selective fluoropeptide (Tyr(3-F)-D-Arg-Phe-Lys-NH$_2$], which is already in use as a research tool to probe the complex opioid receptor system. This unique method of fluorination also permits the facile incorporation of radioactive fluorine into peptides and other biological materials.

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry and Biology of Novel Pyrimidine and Purine Nucleosides

Despite the countless number of modifications of pyrimidines and purines which have been explored in the search for clinically effective and acceptable anticancer and antiviral agents, very few have achieved the conditions required for safe testing in humans. We have undertaken approaches not yet considered by others, in that the unique physical and chemical properties of the fluorine atom are used in key roles.

Uracil condenses specifically with trifluoroacetaldehyde and hexafluoroacetone at C-5 to form derivatives containing perfluoro alcohols at this critical position. Uracil also reacts specifically with bis(perfluoroalkanoyl)peroxides under radical conditions to give perfluoroalkyl derivatives at the same position. The latter compounds lose two fluorine atoms readily at pH 8-9 to form perfluoroalkyl ketones, which exist preferentially as their hydrates or tetrahedral nucleophilic adducts. Since the half-life for loss of fluorine is ca. 20 min. at pH 8, these compounds are potential irreversible affinity labels for pyrimidine recognition sites in vivo. Under the same reaction conditions, the perfluoroalkylpyrimidines are 3-5 times as reactive as trifluoromethylpyrimidines, whose strong antiviral properties have been thoroughly demonstrated. The same radical perfluoroalkylation can be achieved with uridines. Although the carbon-fluorine bond is signficantly more stable in the nucleoside series, conversion to ketones can also be achieved in basic media, particularly with sulfhydryl catalysis. These ketones are reducible to secondary perfluoro alcohols with sodium borohydride. The inductive effects of multiple fluorine atoms renders the alcohols highly acidic and readily capable of forming strong internal hydrogen bonds with a carbonyl or amino group at C-4. We anticipate that such hydrogen bonding will interfere with the intermolecular hydrogen bonding needed for effective base pairing in polynuclear strands and, thus, expect that such compounds may block cell division and may act as antiviral and anticancer agents. Furthermore, hydrogen bonding to this position in purines and pyrimidines has been found essential for the action of PRPP synthetase. By increasing the length of the perfluoroalkyl chain, lipophilicity can be correspondingly increased to meet the needs for effective transport across membranes. The nucleophile at C-4 in uridine or cytidine is also capable of displacing a fluorine atom adjacent to the ketone to form novel bicyclic pyrimidine nucleosides. All of these compounds also have the potential of acting as inhibitors of cytidine deaminase.

Additional studies are under way to introduce analogous perfluoroalkyl groups at C-8 of purines and, thus, provide an additional series of "preaffinity" labels. Our earlier studies on the chemistry of perfluoroalkyl imidazoles indicate that such a group at C-8 of a purine should undergo loss of fluorine under physiological conditions and provide, in vivo, a very reactive functional group capable of bonding to a protein or nucleic acid nucleophile.

SECTION ON BIOCHEMICAL MECHANISMS

Antimalarial Agents Based on Bioheterocycles

Several years ago, we discovered that our novel histidine analogue, 2-fluoro-L-histidine (2-FHIS) is a very potent inhibitor of the growth and cytoadherence of *Plasmodium falciparum*, the malaria parasite which is notoriously resistant to chemotherapy. The organism has the unusual property of inducing production, within an invaded erythrocyte, of a protein containing 30-70% histidine. The protein is found in knobs which are seen on the erythrocyte surface. The high histidine content would create centers of high positive charge; thus, the knobs may be responsible for a very strong adherence of the infected erythrocyte to capillary endothelium, thereby sequestering parasitized cells which would normally be destroyed during passage through the spleen. We had initially selected 2-FHIS for testing because we had demonstrated that 2-FHIS can partially replace histidine in *de novo* protein in both *E. coli* and mammals. Furthermore, the fluorine atom reduces the pK of the imidazole ring from its normal value of 6 to 1.5; therefore, its incorporation into knobs should dilute the clusters of positive charge and reduce cytoadherence. In cultures of infected erythrocytes, low concentrations of 2-FHIS not only do inhibit cytoadherence but prevent maturation of the parasite and the appearance of knobs entirely. The assumption that these antiparasitic properties are due to the incorporation of 2-FHIS into the histidine-rich protein proved unwarranted, since the treated parasite shows a rather low incorporation of labeled 2-FHIS but a general decrease in protein synthesis. As one of several hypotheses for the mechanism of action, we propose that 2-FHIS interferes with histidine on the outer cell surface as a promoter of the transport of some other essential amino acids into the cell. This hypothesis is supported by our earlier findings that 2-FHIS inhibits protein synthesis in cell and organ cultures but not in cell-free systems. Related studies are in progress on the effect of 2-FHIS on facilitated amino acid transport. Unfortunately, the high antimalarial activity shown by 2-FHIS in vitro could not be extended, because the compound proved too toxic to owl monkeys. Such toxicity is surprising, since mice tolerate as much as 500 mg/kg. We are exploring derivatives of 2-FHIS in the hope of reducing toxicity, although the cost of manufacturing any fluorinated bioimidazole would be prohibitive.

Therefore, we have screened a large number of other substituted histidines (which we had synthesized for the first time) for in vitro antimalarial activity: 2-iodo showed very strong and 2-azido gave moderate activity. Neither 4-X-histidines nor 2,4-di-X-histidines show any activity, while 2-iodohistamine is somewhat active. Surprisingly, the 2-chloro and 2-bromo analogues are inactive. Thus, the role of the iodine atom cannot be electronic but may be just the right size to plug the erythrocyte membrane diffusion hole present in infected cells. In contrast to 2-FHIS, 2-IHIS dose not signficantly retard protein synthesis while retarding maturation, a result supporting this mechanism of action. Our 13-C NMR studies show that 2-IHIS has the same NH-tautomer preference as histidine, while 2-BrHIS and 2-ClHIS may have the opposite or unnatural tautomer preference. Thus, the specificity of 2-IHIS may be due to a remarkable tautomer preference which challenges explanation. Although 2-IHIS proved nontoxic to monkeys, it retarted growth of parasite for only 24 h. We suspected that the compound may be a substrate for a mammalian deiodinase but, in the course of a search for such a deiodinase, found that the iodine is rapidly removed by any sulfhydryl reagent or metabolite under physiological conditions. This loss of activity would not be observed in vitro because of the very low levels of sulfhydryl compounds present in the culture dish. The finding that the deiodination need not be enzyme-mediated greatly reduces the possibility of stabilizing the drug by bulky N-alkylation of the imidazole ring or by use of the D-histidine series.

Another approach, therefore, involves efforts to replace the iodine by metabolically stable groups of comparable size; iPr, tBu, Ph, Bz, etc. The most direct route to 2-alkylhistidines is based on a procedure we had developed to synthesize 2-perfluoroalkylhistidines-ring closure of 1,2-diacylaminoethylenes. Initial attempts to apply the same scheme for the introduction of alkyl or aryl groups did not succeed because of decomposition at the high temperatures required. We are now utilizing specific catalysts in order to facilitate these ring closures at lower temperatures.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31100-26 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Laboratory of Bioorganic Chemistry

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	2.7	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Natural products provide a wide range of biologically active agents, many of which have unique profiles of pharmacological activity and therapeutic potential. Over two hundred alkaloids have been identified in extracts from amphibian skins. These included batrachotoxins, which are potent activators of sodium channels, histrionicotoxins, which are noncompetitive blockers of nicotinic receptor-channel complexes and of potassium channels, and pumiliotoxins, which have myotonic and cardiotonic activity due to inhibitory effects on closing of sodium channels. Further alkaloids included new 2,5-disubstituted decahydroquinolines, 5,8-disubstited indolizidines, 1,4-disubstituted quinolizidines, 3,5-disubstituted pyrrolizidines, pumiliotoxins, homopumiliotoxins and allopumiliotoxins, and tricyclic alkaloids, including pyrrolizidine oximes related to nitropolyzonamine a defensive alkaloid in millipedes, cyclopenta[b]quinolizidines, cocinellines identical to defensive alkaloids in ladybug beetles, and the potent nonopioid analgetic epibatidine. Many of these alkaloids had activity as noncompetitive blockers at muscle-type and ganglionic-type nicotinic receptor-channels. Characterization of alkaloids in non-dendrobatid amphibians indicated that the biosynthetic pathways to certain dendrobatid alkaloids have evolved separately in one lineage (genus) of amphibians from the families Bufonidae, Myobatrachidae and Ranidae and in a lineage leading to four genera in the family Dendrobatidae. The lack of alkaloid production in captive-raised dendrobatid frogs remains an enigma although frogs raised in outside vivariums on wild-caught insects did produce limited amounts of alkaloids. Tetrodotoxin-like, and ouabain-like compounds were detected in skin extracts, based on antagonism of radioligand binding, and have been partially characterized. Secretions of a tree frog used by Indians in hunting magic contained a unique peptide, which was named adenoregulin based on its stimulatory effects on binding of agonists to A1-adenosine receptors. Subdomains of an alkaloid-binding site were proposed to explain the interactions of batrachotoxins, aconitine and pumiliotoxin B at sodium channels.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 31101
- 22 LBC

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pharmacology and Metabolism of Biogenic Amines and Related Compounds

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COOPERATING UNITS *(if any)*

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LAB/BRANCH Laboratory of Bioorganic Chemistry

SECTION Section on Pharmacodynamics

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TOTAL-MAN YEARS 0.7	PROFESSIONAL: 0.7	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (b) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The biochemistry, physiology, and pharmacology of biogenic amines, amino acid precursors and metabolic products, and various synthetic derivatives have been investigated. The general areas of study include the effects of fluorine substitution on the properties of biogenic amines, adrenergic antagonists and amino acids and catechol-O-methyltransferase (COMT). Studies specific to fluorine substituted compounds include 1) determination of the adrenergic properties of 2- and 6-fluoro derivatives of dopamine, fluoro-alprenolols, fluoro-practolols 2) mechanism of toxicity of 6-fluoro- and 2,6difluorophenylalanine, 6-fluoro-, 2,3-, 2,5-, 3,5-, 2,6-difluorotyrosine and 2-azidotyrosine in cultured pheochromocytoma (PC12) cell lines. Studies on COMT include: 1) immunohistochemical localization of COMT in the luminal and glandular epithelium of rat uterus during estrus, diestrus, pregnancy, pseudopregnancy, postpartum and following ovariectomy in animals treated with and without exogenous progesterone or progesterone-receptor antagonists 2) localization of COMT and macrophage-specific antigens in macrophages of corpora lutea of rat 3) localization of COMT in the rodent and human intestinal track 4) localization of the site of O-methylation in the dental pulp of rat 5) substrate specificity and reaction kinetics of a series of 3'-hydroxycoclaurines and 3'-hydroxy-N-methylcoclaurines as putative precursors of mammalian opioids 6) confirmation of the primary sequence of peptides isolated from the 23KD form of of rat liver soluble COMT and preparation of monoclonal antibodies from COMT-specfic peptides. Other studies include the irreversible inhibition of brain monoamine oxidase A and B by the binding the isothiocyanates of procaine and proparacaine and the effect of nerve growth factor (NGF) on the uptake of calcium and release of adrenergic neurotransmitters in PC12 cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31102-20 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ion Channels, Receptors and Second Messengers in the Nervous System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	P. Holbrook	NRC Fellow	LBC, NIDDK
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.2	3.7	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Calcium, sodium, potassium, and magnesium ions after translocation through ion channels or by transport proteins can cause activation of release processes, contractile proteins, adenylate and guanylate cyclase, phosphodiesterases, protein kinases, phospholipases, ATPases and other enzymes. Receptors of various types and various toxins serve to modulate ion channels and generation of second messengers. Maitotoxin (MTX), a ladder-like polyether of molecular weight 3424, activated calcium uptake and phosphoinositide breakdown in all cells studied to date. Activation of phospholipase C was dependent on extra cellular calcium and it appeared that MTX in some way enhanced calcium access to the phospholipase. A blocker of receptor-mediated calcium entry, SK&F 96365 antagonized in a similar dose-response relationship MTX-elicited calcium influx, MTX-elicited phosphoinositide breakdown and MTX-elicited insulin release in cultured cells. It appears that the primary effect of MTX is to activate directly the SK&F 96365-sensitive so-called receptor-mediated calcium entry system and, thereby, increase internal calcium, activate phosphoinositide breakdown and trigger release of hormones and neurotransmitters. Development of a radio-labelled MTX for binding studies has been thwarted due to high levels of nonspecific binding. Analogs of MTX lacking sulfate groups acted either as weak activators or as apparent competitive antagonists with respect to calcium uptake and phosphoinositide breakdown. The effects of MTX on phospholipases appeared relatively selective for phospholipase C, but as yet cannot be demonstrated in membranes or permeabilized cells. The effects of amphiphilic peptides mellitin and mastoparan were compared to those of MTX. The potencies were MTX >> melitlin >> mastoparan with respect to calcium influx. All three stimulated phosphoinositide breakdown, while only mellitin and MTX stimulated arachidonate release. The hydrolysis of phosphatidylcholine by phospholipase-D occured with P-O bond cleavage, providing a potential method using oxygen-18 water to assess the pathways for formation of phosphatidic acid in intact cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31104-23 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

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COOPERATING UNITS (*if any*) A. Conney, Rutgers U. (Newark, NJ); W. Levin and A. Wood, Roche Research Center (Nutley, NJ); D. Whalen, Dept. of Chem., Univ. of MD (Catonsville); D. Boyd, Dept. of Chem., Queen's Univ. of Belfast (N. Ireland); R. Lehr, Dept. of Chem. Univ. of Oklahoma (Norman,OK): R. Loncharich, DCRT, NIH; H. Yeh, LAC, NIDDK

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	5	1

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type Do not exceed the space provided.*)

The primary goal has been the elucidation of the structures of reactive metabolites responsible for the carcinogenic, cytotoxic and mutagenic activity of drugs, polycyclic aromatic hydrocarbons, and other environmental chemicals. The approach taken consists of: i) synthesis of primary and secondary oxidative metabolites, ii) study of the metabolism of the chemicals with liver microsomes and with purified cytochromes P450 and epoxide hydrolase, iii) evaluation of the mutagenicity and tumorigenicity of the synthetic metabolites, iv) elucidation of the roles of the cytochrome P450 system and epoxide hydrolase in modulating the mutagenicity of these metabolites, v) determination of the rates and products of reactions of arene oxides and diol epoxides with biopolymers and model compounds, and vi) search for agents capable of preventing the tumorigenicity of reactive metabolites. Synthetic studies have now made available optically pure arene oxide as well as cis and trans dihydrodiols at the 5,6-position of the environmental carcinogen quinoline. P. putida (UV4) forms the cis-(5R,6S)-dihydrodiol while liver microsomes form highly optically enriched (5R,6S)-oxide and (5R,6S)-dihydrodiol on metabolism of quinoline. Acid-catalyzed solvolysis studies of substituted benz[a]anthracene 5,6-oxides as well as theoretical calculations (molecular mechanics by PCMODEL-PI and ab initio by GAUSSIAN 86 and 88 programs) of carbocation stability indicate the importance of steric factors in determining relative reactivity. These data are to be used in evaluation of the mechanism(s) by which microsomal epoxide hydrolase detoxifies such toxic and mutagenic substrates. We have recently proposed an empirical rule for prediction from CD spectra of absolute configuration of nucleoside adducts formed by reaction of the exocyclic amino groups of purine bases at the benzylic epoxide carbon of bay-region diol epoxides. Application of this rule has allowed correction of the assignment of configuration for two benzo[a]pyrene 7,8-diol 9,10-epoxide adducts previously misassigned. Solvolytic studies of the noncarcinogenic DE-1 and carcinogenic DE-2 diastereomers of benzo[a]pyrene 7,8-diol 9,10-epoxides suggest differences in their mechanism of reaction with biochemical nucleophiles at neutral pH (carbocation capture vs nucleophilic attack on neutral epoxide, respectively).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK31106-04 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Mechanistic Enzymology of HIV Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name. title, laboratory and institute affiliation)*

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	J.E. Tropea	IRTA Fellow	LBC, NIDDK
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LAB/BRANCH

Laboratory of Bioorganic Chemistry

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	3.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Methods of enzymology, chemical and enzymatic kinetics, and synthetic, physical and analytical chemistry are being used to investigate the mechanisms of action of reverse transcriptase (RT) and protease enzymes of HIV-1, with the ultimate goal of developing specific inhibitors for these enzymes. Optical assays for retroviral proteases and RTs, developed in this laboratory, are being employed in studies of the effects of reaction conditions and potential inhibitors on the activity of both RT and retroviral proteases. i) A continuous UV spectrophotometric assay described in a previous Report was used to study the elongation, catalyzed by the Klenow fragment of Escherichia coli DNA polymerase I, of a partially self-complementary "hairpin" shaped oligonucleotide consisting of 35 mononucleotide residues. The sensitivity of the method permits the measurement of incorporation of a single base. ii) Concentrations of sodium chloride up to 4.5 M accelerated the hydrolysis of a chromogenic peptide substrate catalyzed by the protease of avian myeloblastosis virus. Similar effects have been observed by us for the cleavage of a peptide substrate catalyzed by HIV-1 protease, and have also been reported by others for cleavage of several peptides by this enzyme. These salt effects appear to be unrelated to the association of subunits that is required for the activity of retroviral aspartyl proteases, since very similar behavior is observed with the monomeric, mammalian enzyme pepsin. iii) We have identified two amino-acid repeat motifs in RT which are conserved in ten HIV-1 and eight HIV-2 isolates, and which occur in a region of the protein that may be involved in association of the p66 and p51 subunits of this enzyme. One motif is a modified "leucine zipper", whereas the other motif involves five tryptophans. Since the activity of HIV-1 RT has been suggested to require subunit association, an understanding of this process may lead to novel approaches to the inhibition of this enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31107-04 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mass Spectrometry of Drugs, Metabolites, Natural Products and Macromolecules.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	L.K. Pannell	Visiting Scientist	LBC, NIDDK
Others:	Y. Murata	Special Volunteer	LBC, NIDDK
	G.J. Wright	Special Volunteer	LBC, NIDDK
	B. Clark	Special Volunteer	LBC, NIDDK
	D.M. Jerina	Section Chief	LBC, NIDDK
	J.W. Daly	Laboratory Chief	LBC, NIDDK

COOPERATING UNITS *(if any)* Becker, Whittaker and White, LAC, NIDDK; Basile and Guo, LNS, NIDDK; Fales, Mason and Jones, LBC, NHLBI; Munro and Blunt, U. Canterbury, New Zealand; Hanbauer, LCP, NHLBI; Mason, Oregon State U., Corvallis, OR; M. Boyd, NCI, FCRC; S. Stein, Natl. Inst. of Stand. and Tech.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Specialized mass spectrometry analyses are provided to the laboratory and to other collaborating units. The emphasis has been primarily devoted to trace organic compounds isolated from biological sources. A very close working relationship has always been maintained with the Laboratory of Biophysical Chemistry, NHLBI. The Californium plasma desorption instrument has been modified to allow UV matrix-assisted ionization. The Ion Trap mass spectrometer is being adapted to allow for higher resolution and to extend its mass range. With this added potential and the mass spectrometry interest at NIH, half the emphasis and effort of this project has become the structural determination of macromolecules, whether through molecular weight determination or sequencing. In line with this change, a formalized collaboration between NHLBI and NIDDK has been established to pursue and develop techniques in this area. This year, major projects have involved the examination of attractants and repellents in snake skins, the objective being to use these to differentiate species or control snake habitat/population (of particular interest was the Brown Tree snake infestation in Guam); the identification of natural benzodiazepines obtained from brain and an examination of their levels as a function of illness; the identification of biologically active natural products, especially those of interest in the AIDS research and treatment; and the structural determination of macromolecules. Samples analyzed derive from many facilities and researchers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 31108-03 LBC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Adenosine Receptor Agonists and Antagonists

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	L.E. Brackett	IRTA Fellow	LBC, NIDDK
	O. Nikodijevic	Guest Worker	LBC, NIDDK
	W. Padgett	Biologist	LBC, NIDDK
	M. Malcolm	Guest Worker	LBC, NIDDK

COOPERATING UNITS (if any)
S.M. Anderson, Walter Reed Inst., Wash. D.C.; M. Beavan, LC, NHLI; R. Sarges, Pfizer, Groton, CT; R. Olsson, U. So. Fla., Tampa, FL; C. Mueller, U. Tubingen, Germany.

LAB/BRANCH
Laboratory of Bioorganic Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	1.5	1.6

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Adenosine regulates a wide range of physiological functions through interaction with at least two major classes of adenosine receptors. The A1 class of adenosine receptors is inhibitory to adenylate cyclase, while the A2 class is stimulatory to adenylate cyclase. Subclasses of adenosine receptors also occur. Some of these are inhibitory to calcium channels, some are stimulatory to potassium channels, some can activate guanylate cyclase, some can modulate phospholipid, while those of smooth muscle cause relaxation through a poorly defined mechanism. In the central nervous system activation of adenosine receptors caused behavioral depression, while blockade of adenosine receptors caused excitation. Activation by selective agonists of the central A1 and A2 receptors had synergistic depressant effects on locomotor activity in mice. Structure activity relationships at A_1 and A_2 adenosine receptors have been analyzed for various agonists and antagonists using binding and adenylate cyclase assays. The agonists included N^6-substituted 2-chloroadenosines, 5'-substituted adenosine analogs, 3,4-dehydro-NECAs, 8,5'-0-anhydroadenosines and various 2-0-alkoxy and 2-0-aryloxyadenosines. The last class were potent and selective for A2A adenosine receptors. 5'-Methylthioadenosine and 5'-chloroadenosine were found to be agonists at A1 receptors, while having no or only partial agonist activity at A2 receptors. The antagonists included pyrazolopyridines related to the anxiolytic catazolate, 8-substituted xanthines and a wide range of caffeine analogs. Cultured PC12 cells, a widely used model system for investigation of A2 receptors, were found to contain mainly high affinity A2A receptors, but also low affinity A2B receptors. The copresence of two receptors complicated analyses of potencies of agonists and antagonists. Fibroblast 3T3 cells contained only A2B receptors, which exhibited the characteristic low affinity of this receptor for agonists, while affinities for antagonists was similar to affinities at A2A receptors. Certain xanthines inhibited antigen-binding to IgE in mast cells and thereby blocked antigen-induced responses.

PHS 6040 (Rev. 1/84) GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER **Z01 DK** **31109-02 LBC**

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interaction between second messengers.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Fabian Gusovsky	Senior Staff Fellow LBC, NIDDK
	John W. Daly	Chief LBC, NIDDK
	David Soergel	Biologist LBC, NIDDK
	Motohiko Takemura	Visiting Fellow LBC, NIDDK

COOPERATING UNITS *(if any)*

J.S. Gutkind (LCDO, NIDR), A. Weissman (EIB, NCI), W.Z. Potter (NSB, NIMH), P. Skolnick (LN, NIDDK), M. Beaven (LCP, NHLBI), J. Baumgold (G. Washington University, Washington, D.C.).

LAB/BRANCH Laboratory of Bioorganic Chemistry

SECTION Section on Pharmacodynamics

INSTITUE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL-MAN YEARS 2.6	PROFESSIONAL: 1.6	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (b) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interactions between second messenger systems have been characterized in NIH 3T3 cells. Activation of protein kinase C with phorbol esters results in this cell line in a significant reduction in forskolin-induced formation of cyclic AMP. Such interaction is mediated by the γ-isoform of protein kinase C. Two distinct receptors for prostaglandins are present in NIH 3T3 cells. One receptor is coupled to phospholipase C. This receptor has a profile of selectivity of PGF2α>>PGE2. Stimulation of phospholipase C by PGF2α is only partially inhibited by pertussis toxin pretreatment. Experiments in permeabilized cells demonstrate a requirement of a guanine nucleotide for PGF2α stimulation, indicating that activation of phospholipase C involves G protein. The second receptor that interacts with prostaglandins in NIH 3T3 cells causes a stimulation of adenylate cyclase. This receptor has a profile of selectivity of PGF2α=PGE2. At concentrations of 1-10 μM, activation of cyclic AMP formation by PGF2α is significantly lower than that obtained with PGE2. The inhibitory action of high concentrations of PGF2α is attributed to activation of protein kinase C through phospholipase C activation and subsequent formation of diacylglycerol, since in the presence of a phorbol ester, PGE2-induced formation of cyclic AMP is comparable to that attained with high concentrations of PGF2α alone. Overnight treatment of NIH 3T3 cells with PGF2α results in a down regulation not only of prostaglandin-induced stimulation of phospholipase C, but also of guanine nucleotide-induced activation in permeabilized cells. This result suggests that effects on G protein(s)-mediated stimulation of phospholipase C may be partly responsible for the desensitization of receptor-mediated responses observed in these cells. Identification of such G protein(s) would therefore permit molecular characterization of the mechanism of down regulation. With the use of polymerase chain reaction (PCR), the expression of G proteins was examined in NIH 3T3 cells. Sequences corresponding to common regions in previously cloned G proteins were utilized as PCR primers. A sequence corresponding to Gα11, which mediates phospholipase C activation in vitro, was identified in NIH 3T3 cells. Full length cDNAs for coding regions were obtained, introduced into mammalian expression vectors and transfected into NIH 3T3 cells. Transfected cells showed an enhanced response of phospholipase C to PGF2_ when compared to vector-transfected controls. This result provides evidence for Gα11 involvement in phospholipase C activation in the action of PGF2α in NIH 3T3 cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 31110-15 LBC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analogues of Thyrotropin-releasing Hormone

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Others:	Virender M. Labroo	Special Expert (Term 1/91)	NIDDK-LBC

COOPERATING UNITS *(if any)* A. Spatola, Louisville, KY; C. Stammer, Athens, GA; A. Siren, USUHS; S. Vonhof, USUHS; I. Paakari, Helsinki, Finland; A. Faden, San Francisco, CA; Y. Tache, Los Angeles, CA.

LAB/BRANCH
Laboratory of Bioorganic Chemistry

SECTION
Section on Biochemical Mechanisms

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies with mobile rate and binding studies with rat brain homogenates have made it clear that endocrine and various centrally mediated actions of TRH involve uniquely different receptors and that, after decades of effort in various laboratories, we have achieved the separation of these activities with our novel TRH analogues; these analogues all have electronegative groups on the imidazole ring of histidine. Thus, 4(5)-nitro-Im-TRH is highly selective for cardiovascular activity (elevation of heart rate and blood pressure), and may be useful in the treatment of various forms of shock without any endocrine effects. On the other hand, Nva(2)-TRH is a selective analeptic agent without effect on the cardiovascular system; this analogue has served as a research tool for delineation of those binding sites in rat brain which may mediate the analeptic effects of TRH and its other analogues.

Computer-assisted structure-activity analysis of a large number of imidazole-substituted analogues has helped us design more selective and more potent members, as well as photoaffinity labels for TRH receptors. Thus, our predictions of the vital role of imidazole-NH tautomer preference in determining selectivity in binding by brain receptors has been validated by the demonstration that 1-Me-5-nitro-Im-TRH binds ca. 3000 times as tightly as 1-Me-4-nitro-Im-TRH. Recently, we have carried out receptor-binding analysis of various TRH analogues with subtle backbone modifications (replacement of the peptide amide with a thioamide surrogate); these studies have allowed us to identify subtle differences in the high affinity TRH receptors in rat pituitary and brain. Current efforts are devoted to the synthesis and evaluation of analogues of TRH designed to meet the requirements of our new concept of "receptor-activated affinity labels" (RAAL's). Ideally, such a compound would be able to survive the onslaught of nucleophiles in serum and tissues, and yet be able to react selectively and irreversibly with a nucleophile in the appropriate receptor environment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31111-21 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stereopopulation Control in Drug Delivery and Enzyme Simulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Michael M. King	Special Volunteer	GWU

COOPERATING UNITS (if any) Y. Ueno, Nagoya, Japan; W. Antkowiak, Poznan, Poland; Y. Takeuchi, Toyama, Japan; W. Dürckheimer, Frankfurt, FRG; M. Impicciatore, Parma, Italy; J. Flippen-Anderson, NRL, Washington, DC

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER.
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Enzymes accelerate organic chemical reactions by 10-20 powers of ten over their closest test-tube counterparts. In order to account for this remarkable catalytic power of enzymes, it is usually considered that the activation free energy is contributed both by binding of the substrate to the enzyme (step 1) and by chemical transformation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2. We have proposed, however, that the overall catalytic process is more easily justified on the assumption that the first step contributes a more significant share of the activation energy than is generally accepted. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions (independently of any functional assistance) at rates comparable to those catalyzed by enzymes, and show that the protein raises both the entropic and enthalpic components of the substrate by binding it in a single, rigid conformation. Recent work has involved a study of steric and electronic effects on NMR and IR spectra across tight space rather than through covalent bonds. These studies show that kinetic and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limit of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo group. After considerable effort, this goal has been reached and further work is in progress. As part of our studies of practical application of stereopopulation control, we have been exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport from the gut to the circulatory system to the brain by the temporary masking of charge within the molecule, by improvement in lipophilicity and by regeneration based simply on local pH variation in receptor sites.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31112-15 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991 -

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Chemistry of Bioimidazoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Virender Labroo	Special Expert (Term 1/91)	NIDDK-LBC
	Albinus D'Sa	Visiting Fellow	NIDDK-LBC
	Ellen Goldberg	IRTA Summer Fellow	NIDDK-LBC
	Tuan Tran	Stay-in-School	NIDDK-LBC
	Gopal Nand	Special Volunteer (Summer)	FAES
	Felix Stephenson	Special Volunteer (Summer)	FAES

COOPERATING UNITS (if any) H. Kimoto, Nagoya, Japan; M. Nishida, Nagoya, Japan; E. DeClercq, Louvain, Belgium; A. Shanzer, Rehovot, Israel; W. Nagai, Nagoya, Japan; J. Retey, Karlsruhe, FRG; W. Dürckheimer, Frankfurt, FRG; S. Avramovici, Jerusalem, Israel

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	0.7	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Perfluoroalkylimidazoles have attracted considerable interest because of their utility in providing lipophilic sites in medicinal agents and because of their potential use as "receptor-activated affinity labels" (RAAL's) for irreversible blocking of receptors in chemotherapy. The simplest and most general methods for perfluoroalkylation of all heterocyclic rings have been developed in this laboratory and are based on the photochemical generation of perfluoroalkyl radicals. Photochemical perfluoroalkylation of imidazoles leads to mixtures of 2- and 4-isomers while the reaction with pyrroles occurs only at C-2. The practicality of the method for complex substrates has been demonstrated by the facile trifluoromethylation of histidine in peptides (e.g., TRH), followed by HPLC separation of the isomers. Exposure of the trifluoromethyl derivatives of TRH to aqueous ammonia leads to their conversion to the corresponding cyano derivatives. All of these derivatives show very selective binding to low affinity TRH receptor sites in the brain. We have now developed a procedure for regiospecific substitution by blocking all undesired sites with the methylthio group, which is subsequently removed with Raney nickel. Thus, 3-perfluoroalkylpyrroles have been made for the first time. Perfluoroalkylimidazoles have been found to undergo facile nitration and halogenation without destruction; therefore, methods are now available to prepare photosensitive ligands (azido, diazo) and ligands labeled with radioiodine. We have also synthesized pentafluoroethylhistidine and histamine isomers by photochemical radical substitution. These compounds are converted by base into the corresponding trifluoroacetyl derivatives, which have such reactive carbonyl groups that they may serve as affinity labels for bioimidazole-binding sites. Upon treatment with methanolic base, the trifluoromethyl analogs can be converted into trimethoxymethyl and the pentafluoroethyl derivatives into ketals. These ortho functionalities are also of interest as potential covalent affinity labels.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31113-15 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Halogenated Biogenic Amines in Biochemistry and Pharmacology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Kenneth L. Kirk	Chief	LBC, NIDDK
Others:	Bang-Hua Chen	Visiting Fellow	LBC, NIDDK
	Jun-ying Nie	Visiting Assoc.	LBC, NIDDK
	Fabian Jameison	IRTA Fellow	LBC, NIDDK
	Vital Shetty	Special Volunteer	LBC, NIDDK
	J.W. Daly	Chief	LBC, NIDDK
	C.R. Creveling	Research Chemist	LBC, NIDDK
	Fabian Gusovsky	Sr. Staff Fellow	LBC, NIDDK

COOPERATING UNITS *(if any)* Brian Ciliax (BEIB); M. Channing, D. Kiesewetter, (CC, Dept. of Nuclear Medicine); D.S. Goldstein (HE, NHLBI), I.J. Kopin (DiR, NINCDS); Claire Fraser (NIAAA); M. Linnoila (NIAAA); R.S. Phillips (Univ. of Georgia).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Biogenic amines play key roles in neurotransmission, metabolism, and in control of various physiological processes. Using a variety of synthetic methodologies, including novel procedures developed by us, we have prepared a series of biogenic amines with fluorine substituted at various ring-positions. By virtue of its very small size and high electronegativity, fluorine is a very favorable replacement for hydrogen in these analogs. The biological properties and usefulness of these ring-fluorinated biogenic amines have proved to be extremely rewarding and continue to find applications in a multitude of studies, including research on the mechanisms of transport, storage, release, metabolism, and modes of action of these amines. Of particular significance was the discovery that 6-fluoronorepinephrine is a selective α-adrenertic agonist and 2-fluoronorepinephrine is a selective β-adrenergic agonist. Mechanisms considered to explain these results include: 1) a direct effect of the C-F bond on agonist-receptor interaction or 2) an indirect effect of the C-F bond on the conformation of the ethanolamine side-chain. The results of testing of new analogs synthesized to probe these mechanisms indicate that electronic effects may be more important than conformational factors. Fluorinated analogs are useful biological tracers. For example, [^{18}F]-labeled 6-fluorodopamine, the biological precursor to 6-fluoronorepinephrine, has been found to be an excellent PET-scanning agent for peripheral noradrenergic innervation. Fluorinated products of metabolism of 6-fluoronorepinephrine, 6-fluorodopamine, and 6-fluoro-Dopa have been prepared to aid in development of these PET-scanning agents. A difluoromethylenephosphonate analogue of AZT has been prepared and found to be active against HIV-reverse transciptase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31114-09 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Significance of Ligand Tautomerism in Biorecognition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Others:	Rita Labroo	Visiting Associate (Term 2/91)	NIDDK-LBC
	Edith Miles	Senior Investigator	NIDDK-LBP
	Virender Labroo	Special Expert (Term 1/91)	NIDDK-LBC
	Catherine Dong	Visiting Fellow (EOD 7/91)	NIDDK-LBC

COOPERATING UNITS *(if any)* Robert Phillips, Athens, GA; H. Kimoto, Nagoya, Japan; J. Flipper-Anderson, NRL, Washington, DC; W. Dürckheimer, Frankfurt, FRG; Shelly Avramovici, Jerusalem, Israel; M. Impicciatore, Parma, Italy

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A large variety of metabolically important molecules exist as pairs of tautomers, in which the major tautomer (tantotautomer) is so favored energetically over the minor tautomer (tenutautomer) that the latter species cannot even be detected in the equilibrium mixture by any available spectroscopic means. Nevertheless, mechanistic studies have revealed that the tenutautomer is very often the "true" reactant in various chemical transformations. We have proposed that the same phenomenon may exist in biological systems, namely, that the tenutautomers of various metabolites may be the "true" ligands for some enzymes and receptors. This theory provides a new pathway for substrate activation; it also leads to the critical consequence that potential inhibitors should be designed as analogues of the tenutautomers, rather than the common practice of mimicking the tantotautomers. Since it is still quite impossible to examine the structure or conformation of a ligand within a binding site, arguments for the tenutautomer hypothesis must be based on evidence and inference from the behavior of stable analogues of the tenutautomer.

This approach was highly successful and convincing in the case of tryptophan. We found that stable analogues of the indolenine tautomer having a tetrahedral carbon at C-3—e.g., 2,3-dihydro-L-tryptophan, oxindolyl-L-alanine and dioxindolyl-L-alanine—are potent competitive inhibitors of tryptophan synthase and tryptophanase, enzymes involved in the biosynthesis and degradation of tryptophan. Further, the two enzymes show "mirror-image" specificity, in that the 3R diastereoisomer of each analogue inhibits only tryptophanase, while the 3S diastereoisomer inhibits only tryptophan synthase. A logical extension of this series involves the syntheses of 3-azido analogues as photoaffinity labels, as well as tetrahedral analogues of 5-hydroxy and 5-methoxy bioindoles.

Additional work is in progress on the synthesis of tenutautomer analogues of tyrosine and dihydroxyphenylalanine (DOPA), as well as analogues of histamine and histidine. The latter compounds are expected to inhibit histidine deaminase and urocanase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31115-08 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Functionalized Congeners of Bioactive Compounds

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.	K. Jacobson	Research Chemist	LBC, NIDDK
Others:	X. Ji	Visiting Assoc.	LBC, NIDDK
	P. Van Galen	Visiting Fellow	LBC, NIDDK
	N. Melman	Special Volunteer	LBC, NIDDK
	G. Pinder	Special Volunteer	LBC, NIDDK

COOPERATING UNITS *(if any)*

J. Baumgold (GW Univ.); B. Madras (Harvard Univ.); G. Stiles (Duke Univ.); Y. Karton (Israel Inst. Biological Research, Nes Ziona, Israel); E. Heilbronn (U. Stockholm); H. Pollard (LCBG, NIDDK).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Recent work in our laboratory has demonstrated that certain drugs may be attached to well-defined "carrier" molecules and still retain the ability to bind to the receptor site and effect biological activity. This synthetic strategy for the attachment of drugs to carriers is termed the "functionalized congener" approach. The "carrier" molecule may be many times larger than the parent drug; indeed there is practically no maximum size limitation for a fully potent analog. Unlike the prodrug approach or the immobilization of drugs for slow release, the "functionalized congener" approach is designed to produce analogs for which no metabolic cleavage step is necessary for activation. Moreover, the attachment of the drug to ~~a~~ "carrier" such as a peptide may result in the enhanced affinity at an extracellular receptor site and an improvement in the pharmacological profile of the parent drug. The A_1-adenosine receptor from bovine brain was purified to homogeneity by affinity chromatography using an immobilized xanthine functionalized congener.

This strategy is being employed in the design of new agonists and antagonists at muscarinic acetylcholine receptors and at P_2 purinergic receptors. These ligands are synthesized and characterized and then screened for potency and selectivity in binding assays and in functional assays. We have probed sites for attachment of "carrier" molecules to pirenzepine, an M_1-muscarinic antagonist that is used in the treatment of gastric ulcers. A particular site for amino-alkyl chain derivatization that enhances biological potency was found.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31116-04 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prosthetic Groups for Radiolabeling of Functionalized Drugs and Peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	K. Jacobson	Research Chemist	LBC, NIDDK

COOPERATING UNITS *(if any)*

M. Lesniak (NIDDK); R. Eastman (NIDDK); M. Channing (NM-CC); G. Stiles (Duke University).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The use of radioisotopes to label organic compounds for use in diagnostic nuclear medicine is well documented in the literature. It has been found that certain radiolabeled compounds will localize in the brain, heart, or in other target organs or tissues to a sufficient level to allow for imaging thereof. There has been increasing interest in finding compounds which will more effectively cross the blood-brain barrier, thus facilitating more efficacious imaging of the brain.

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabeled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as 18F, 123I, and 125I, to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor, or drugs that bind the label irreversibly (trifunctional reagents). Positron emission tomography (PET) has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of 18F requires rapid and efficient reaction and purification; since the half-life is only 110 minutes. We are utilizing this approach to image insulin receptors in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31117-04 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Drugs Acting at Adenosine Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	K. Jacobson	Research Chemist	LBC, NIDDK
Other:	X. Ji	Visiting Assoc.	LBC, NIDDK
	P. Van Galen	Visiting Fellow	LBC, NIDDK
	M. Maillard	Special Volunteer	LBC, NIDDK
	M. Glashofer	Special Volunteer	LBC, NIDDK

COOPERATING UNITS *(if any)* P. Churchill (Wayne State); G. Stiles (Duke University); L. Wang (University of Alberta), R. Bartus (Cortex Pharmaceuticals); R. Green (University of Illnois); K. Lee (U. Virginia); L. Belardinelli (U. Florida).

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Section on Drug Receptor Interactions

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	2.1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The extracellular adenosine receptor has a modulatory role in the nervous, circulatory, endocrine, and immunological systems. The prospect of harnessing these effects specifically for therapeutic purposes is attractive.

A functionalized congener approach to drug design in which a "carrier" molecule is attached to a receptor pharmacophore has been applied to the adenosine receptor to produce analogs of agonists and antagonists which have promise as therapeutic agents and as receptor probes. Prodrugs of adenosine agonists and antagonists targeted for the brain and kidneys are being designed. In the antagonist series new analogs which combine potency, water solubility, and A_1-adenosine receptor selectivity in the same compound are now being evaluated in in vivo testing. In the central nervous system adenosine agonists act as cerebroprotective agents. We are developing new agonists as potential agents for treating stroke. Purine functionalized congeners are also being developed as adenosine receptor probes. An iodinated adenosine derivative, selective for the A_2 subtype, was used to characterize the carbohydrate groups that are attached to the native receptor. Rabbit brain A_2-receptors undergo a specific proteolysis that alters pharmacological properties.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31118-02 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Aldose Reductase Inhibitors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Others:	Rita Labroo	Visiting Associate (Term 2/91)	NIDDK-LBC

COOPERATING UNITS (if any)

Peter Kador, LMOD, NEI; J. Flippen-Anderson, NRL, Washington, DC

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Efforts to retard and ameliorate late-onset diabetic complications have been directed toward reversible and/or irreversible inhibition of the enzyme, aldose reductase. These complications affect the eye (cataracts), kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of abnormally high intracellular concentrations of sorbitol, in turn resulting from the reduction of excess glucose by aldose reductase. Our studies on the synthesis of inhibitors of tryptophan-metabolizing enzymes have produced oxindole derivatives which are somewhat analogous in overall geometry to certain commercial compounds which had reached advanced clinical trials as aldose reductase inhibitors. In vitro assays have revealed that, while these oxindole derivatives (lactones) are not significantly active, their hydrolysis products are as potent as any others thus far developed. Although these chiral compounds are not amino acids, we have succeeded in the use of chymotrypsin to achieve their resolution by selective ester hydrolysis. Assignment of stereochemistry has been achieved through x-ray crystallography and through the novel approach of destroying the known chiral center while retaining the unknown center in a diastereoisomer. The introduction of certain substituents on the aryl ring of the oxindole has been found to increase inhibitor activity several fold. Additional analogues have been prepared which are expected to serve as irreversible affinity and photoaffinity labels for the enzyme. Current efforts are being devoted to improvements in yield, in resolution and in enhancement in lipophilicity, with the goal of achieving more effective penetration and transport to the sites of action in vivo. Yet another new direction involves replacement by sulfur of the oxindole oxygen, in order to generate a trigonal carbon at C-3. Thus, we hope to learn more about structure-activity requirements. Since our inhibitor series is significantly different in structure and in functional groups from those which had reached clinical trials, we have strong hope that this series will not elicit the serious side effects which ultimately had caused many of the commercial candidates to be abandoned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31119-02 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Others:	Virender Labroo	Special Expert (Term 1/91)	NIDDK-LBC
	Albinus D'Sa	Visiting Fellow	NIDDK-LBC
	Catherine Dong	Visiting Fellow (EOD 7/91)	NIDDK-LBC
	Tuan Tran	Stay-in-School (EOD 5/91)	NIDDK-LBC

COOPERATING UNITS (if any) J. Flippen-Anderson, NRL, Washington, DC; S. Vonhof, USUHS; W. Dürckheimer, Frankfurt, FRG

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Introduction of conformational restraints in peptides is a very useful means to probe peptide-receptor interactions and to enhance potency and/or selectivity. While local conformational restraints can be imposed by incorporation of unsaturated or small ring amino acids, side chain-side chain cyclization to form cyclic analogues has proved to be one of the most useful approaches for the introduction of more general conformational constraints: e.g., a disulfide bond between Cys or Pen residues, or an amide bond between side chains of amino and carboxy triflunctional amino acids. The 13-member cyclic peptide, H-Tyr-D-Orn-Phe-Asp-NH_2 (cyc-Orn-Asp), is a potent μ-selective opioid peptide. Molecular modeling of this peptide has revealed great flexibility in the ring structure, making it difficult to identify a single conformation which may be biologically relevant. In order to restrict further the conformational mobility in such peptides, we have designed and synthesized a novel trifunctional amino acid based on pyrrolidine (PDA). This amino acid, if used in place of Orn or Lys for side chain-side chain cyclization, is expected to reduce considerably the conformational flexibility in the macrocyclic ring of these peptides owing to the introduction of a cyclic structure. Moreover, the additional asymmetric center in the pyrrolidine ring may introduce differential stereochemical constraints to the binding of diastereoisomeric peptides, derived from the corresponding diastereoisomeric PDA's. Thus, these peptides may act as probes for delineating the stereochemical topology of the receptor in the vicinity of the ring structure and may have useful biological properties and applications. Optically active amino acids are being used increasingly as synthons for the preparation of a variety of chiral compounds. PLA's can be expected to be excellent synthons for compounds such as pyrrolizidines with the stereochemistry already defined at two centers. Furthermore, these amino acids may possess antimicrobial or other antimetabolic activities. An efficient method for the synthesis of all four optically active stereoisomers of PDA has been developed. These amino acids will be used for the preparation of novel bicyclic opioid peptide analogues.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31120-02 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fluorinated Analogues of Bioactive Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Others:	Virender Labroo	Special Expert (Term 1/91)	NIDDK-LBC
	David Hebel	Visiting Fellow (Term 10/90)	NIDDK-LBC
	Albinus D'Sa	Visiting Fellow	NIDDK-LBC
	Kenneth L. Kirk	Chief, Section on Drug-Receptor Interactions	NIDDK-LBC

COOPERATING UNITS (if any) P. W. Schiller, Montreal, Canada; W. Dürckheimer, Frankfurt, FRG; Shaw Chen, CH, LMG

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Laboratory of Bioorganic Chemistry

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Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Fluorinated analogues of many bioorganic compounds have become important pharmacological and medicinal agents. The advantages of fluorine substitution arise from its small van der Waals radius (1.35 A). As a result, fluorine bonded to a trigonal carbon is used effectively as a C-H replacement. Because of similar aliphatic C-F and C-O bond lengths (1.39 and 1.43 A, respectively), the halogen bonded to a tetrahedral carbon has been used frequently as an OH surrogate, particularly in studies with nucleoside analogues. These steric similarities allow fluorinated compounds to mimic their nonfluorinated parents with respect to recognition by various biological macromolecules such as enzymes, transport proteins and receptors. On the other hand, the high electronegativity of fluorine (4.0) can drastically alter electron density distribution in the molecule which, in turn, affects pK values in neighboring groups and molecular dipole moments. All these altered physicochemical properties, as a result of fluorine substitution, can result in drastically modified biological properties such as potency, transport and receptor selectivity. Furthermore, recent advances in 19-F NMR and electron energy-loss spectroscopic techniques, and 18-F positron-emission tomography (18-F PET), have increased the significance and application of fluorinated molecules as biological markers and diagnostic agents.

Although many methods have been developed for rapid and efficient incorporation of 18-F and 19-F into various molecules, such methods have not been generally practical for peptides. We have now developed a method for fast, efficient introduction of fluorine regiospecifically into the phenolic ring of tyrosine-containing peptides by use of the electrophilic fluorinating agent, acetyl hypofluorite (AcOF). We have used this method for fluorinating μ-selective opioid peptides. In vitro bioassays and receptor-binding assays have revealed that these fluoro peptides retain their high receptor selectivity. Thus, we have been able to prepare a very μ-selective fluoropeptide [Tyr(3-F)-D-Arg-Phe-Lys-NH_2], which is already in use as a research tool to probe the complex opioid receptor system.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31121-01 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry and Biology of Novel Pyrimidine and Purine Nucleosides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Albinus D'Sa	Visiting Fellow	NIDDK-LBC
	Tuan Tran	Stay-in-School	NIDDK-LBC

COOPERATING UNITS (if any) H. Kimoto, Nagoya, Japan; M. Nishida, Nagoya, Japan; H. Sawada, Ibaraki, Japan; E. DeClercq; Louvain, Blegium; W. Dürckheimer, Frankfurt, FRG

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER.
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Uracil condenses specifically with trifluoroacetaldehyde and hexafluoroacetone at C-5 to form derivatives containing perfluoro alcohols at this critical position. Uracil also reacts specifically with bis(perfluoroalkanoyl)peroxides under radical conditions to give perfluoroalkyl derivatives at the same position. The latter compounds lose two fluorine atoms readily at pH 8-9 to form perfluoroalkyl ketones, which exist preferentially as their hydrates or tetrahedral nucleophilic adducts. Since the half-life for loss of fluorine is ca. 20 min at pH 8, these compounds are potential irreversible affinity labels for pyrimidine recognition sites in vivo. Under the same reaction conditions, the perfluoroalkylpyrimidines are 3-5 times as reactive as trifluoromethylpyrimidines, whose strong antiviral properties have been thoroughly demonstrated. The same radical perfluoroalkylation can be achieved with uridines. Although the carbon-fluorine bond is significantly more stable in the nucleoside series, conversion to ketones can also be achieved in basic media, particularly with sulfhydryl catalysis. These ketones are reducible to secondary alcohols with sodium borohydride. The inductive effects of multiple fluorine atoms renders the alcohols highly acidic and readily capable of forming strong internal hydrogen bonds with a carbonyl or amino group at C-4. We anticipate that such hydrogen bonding will interfere with the intermolceular hydrogen bonding needed for effective base pairing in polynuclear strands and, thus, expect that such compounds may block cell division and may act as antiviral and anticancer agents. Furthermore, hydrogen bonding in this position in purines and pyrimidines has been found essential for the action of PRPP synthetase. By increasing the length of the perfluoroalkyl chain, lipophilicity can be correspondingly increased to meet the needs for effective transport across membranes. The nucleophile at C-4 in uridine or cytidine is also capable of displacing a fluorine atom adjacent to the ketone to form novel bicyclic pyrimidine nucleosides. All of these compounds also have the potential of acting as inhibitors of cytidine deaminase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31122-01 LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antimalarial Agents Based on Bioheterocycles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Ellen Goldberg	IRTA Summer Student	NIDDK-LBC
	Gopal Nand	Special Volunteer	FAES

COOPERATING UNITS (if any)

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER.
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The high antimalarial activity we had found for 2-fluorohistidine (2FHIS) against Plasmodium falciparum in vitro could not be extended because the compound proved too toxic to owl monkeys, despite the fact that mice tolerate up to 500 mg/kg. Therefore, we have screened a large number of other substituted histidines (which we had synthesized for the first time) for in vitro antimalarial activity: 2-iodo showed very strong and 2-azido gave moderate activity. Neither 4-X-histidines nor 2,4-di-X-histidines show any activity, while 2-iodohistamine is somewhat active. To our surprise, the 2-chloro and 2-bromo analogues are inactive. Thus, the role of the iodine atom cannot be electronic but may be just the right size to plug the erythrocyte membrane diffusion hole present in infected cells. In contrast to 2-FHIS, 2-IHIS does not significantly retard protein synthesis while retarding maturation, a result supporting the proposed mechanism of action. Although 2-IHIS proved nontoxic to monkeys, it retarded growth of parasite for only 24 h. We suspected that the compound may be a substrate for a mammalian deiodinase but, in the course of a search for such a deiodinase, found that the iodine is rapidly removed by any synthetic or natural sulfhydryl species under physiological conditions. This loss of activity would not be observed in vitro because of the very low levels of sulfhydryl compounds present in the culture medium. The finding that the deiodination need not be enzyme-mediated greatly reduces the possibility of stabilizing the drug by bulky N-alkylation of the imidazole ring or by use of the D-histidine series.

Another approach, therefore, involves efforts to replace the iodine atom by metabolically stable groups of comparable size: iPr, tBu, Ph, Bz, etc. The most direct route to 2-alkylhistidines is based on a procedure we had developed to synthesize 2-perfluoroalkylhistidines—ring closure of 1,2-diacylaminoethylenes. Initial attempts to apply the same scheme for the introduction of alkyl or aryl groups did not succeed because of decomposition at the high temperatures required. We are now utilizing specific catalysts in order to facilitate these ring closures at lower temperatures.

Annual Report of the Laboratory of Molecular Biology

National Institute of Diabetes and Digestive and Kidney Diseases

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research program involves a broad range of experimental approaches to problems in molecular genetics, the regulation of gene expression in prokaryotes and eukaryotes, and the structures of nucleic acids and proteins. Current research includes studies on the organization of DNA and proteins within the eukaryotic nucleus, studies on the molecular mechanisms for establishing and maintaining stable states of gene expression during embryogenesis, studies of site-specific recombination mechanisms in a variety of systems including retroviruses, bacteriophage, and the genes of the immune system, and studies of DNA supercoiling and its regulatory effects as well as studies of antibiotic resistance mechanisms and of metabolic channeling in bacteria. More directly structural research includes studies on the chemistry, structures and interactions of polynucleotides, calorimetric studies of nucleic acids and proteins, studies of molecular crowding in biological systems, and a large number of crystallographic studies of the structures of enzymes, viral proteins, and immunoglobulins. Significant progress has been made in all of these areas in the past year.

Chromatin Structure and Function

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have cloned the gene for the general erythroid-specific factor GATA-1 (Eryf1) of chicken. We find that the promoter of the gene contains binding sites for a number of known trans-acting factors, including a cluster of sites for GATA-1 itself. Transactivation studies show that these sites function in an autoregulatory mechanism. Other features of the promoter suggest mechanisms that may function early in development. We have continued studying the stage-specific expression of globin genes. We find that the Π-globin gene, an embryonic α-family gene, is controlled by a complex mechanism involving three factors that change their concentration at the embryonic-adult switch. We have continued studies of the effects of a β-globin locus control region (LCR or DCR) on gene expression and chromatin structure. The LCR confers position independence of expression on nearby genes. We find that it is also capable of generating a hypersensitive domain in the absence of other elements, while elements like the β-globin promoter are not, a distinction important in understanding how active chromatin is generated. In order to understand how the histones of chromatin function during transcription, we have also studied the effect of positive supercoiling on nucleosome core formation. We find that core particles formed on such DNA are perfectly normal, but spontaneously transfer to negatively supercoiled DNA when that is available. We have proposed a mechanism, based on these observations, for transcription through nucleosome-covered DNA.

Structural Molecular Biology

The position and magnitude of the Zn induced bend within the 5s RNA gene of Xenopus has been determined. The center of the bend is located at about bp +65 within the gene. The bend magnitude is at least 50-60 degrees, comparable to naturally occuring bends from phased A tracts, until now the only other sequence known to bend DNA. These values were obtained by quantitative analysis of changes in rotational diffusion coefficients with and without added Zn for a series of overlapping DNA fragments spanning the 5s RNA gene. Given the specificity of Zn binding for purines, the most probable cause of the bending is due to junction angles between oligopurines tracts with a Zn induced altered conformation and adjacent canonical B-form structures. Both the oocyte and somatic sequences have four of these oligopurine tracts within a 60 bp region. They are symmetrically located about a bp +70 center and have an underlying 10 bp phasing. This bend is found within the binding domain of TF IIIA, a zinc finger protein that plays a key role in regulating 5s RNA gene activity. There is evidence in the literature, as well as our own preliminary results, that TF IIIA binding also bends DNA. The binding strength of TF IIIA to sequence variants of the binding domains seems to correlate with the symmetry and phasing of the oligopurine tracts. The bending of DNA by TF IIIA binding is likely accomplished by stabilizing the same alternate DNA conformation as Zn binding.

Control of Gene Expression during Chicken Erythrocyte Development

Developmental regulation of expression of two major gene families was studied: globin genes and HMG 14(a and b) and 17 genes. The former represents a differentiated function restricted to erythrocytes whereas the latter genes are constitutively expressed as an essential component of cellular chromatin.

A. Globin genes. Expression of the ε globin gene is restricted to the primitive erythrocyte, which circulates only early in chick embryogenesis (day 1-7). The promoter has been sequenced and putative control regions are being analyzed by in vitro footprooting and gel mobility shift assays. The extracts used for these analyses are derived from erythrocytes obtained at different stages of development spanning the time when the ε gene is fully active to the adult chicken, when all genes are totally inactive. These regions of differential binding within the ε promoter are also being analyzed for functional activity by linkage to a reporter gene and subsequent transfection into primary erythrocytes. A special β-globin reporter gene has been constructed for this purpose.

B. HMG 14(a and b) and 17. To establish this system we investigated various parameters related to expression of these genes in erythrocytes during embryogenesis: pulse-labeled protein, steady state mRNA, nuclear run-ons that analyze only newly synthesized transcripts, and chromatin structure of the genes themselves. Our results suggest that the individual genes of the HMG family are differentially expressed in development. HMG 14a, in particular, is restricted to the primitive (early) erythrocyte whereas HMG 14b and 17 are maximally expressed only later, in the definitive erythrocyte, but not in the reticulocyte.

Developmental Regulation of Differential Gene Expression

Our work is focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogenesis. Progress has been achieved in three key areas:

1. We have defined at single base pair resolution the protein-nucleic acid interactions over active and inactive 5S ribosomal RNA genes in vivo. These results strongly support a role for stable transcription complexes in maintaining gene activity and for nucleosomes in maintaining the repressed state.

2. We have defined the structure of DNA in a nucleosome and the contributions of histone domains to that structure. We find that DNA sequences of widely divergent structure in solution adopt the same structure in the nucleosome. Purified transcription factors interact with comparable affinity to their promoters as naked DNA and when associated with histones H3/H4. Histone H2A/H2B, when incorporated into a nucleosome inhibit transcription factor binding. These results provide a molecular explanation for the access of transcription factors to DNA during staged chromatin assembly at the replication fork.

3. We have defined a new DNA binding motif in the Y-box transcription factors. Germ cell selective variants of these factors have been cloned from Xenopus and mouse respectively suggesting a developmental role that has been conserved through evolution.

Studies on the Mechanism of Genetic Recombination

The major objective of this project is to uncover the enzymatic steps involved in various genetic rearrangement reactions and to study the mechanism of action of the enzymes involved. The mechanism of the transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate a branched DNA intermediate. Efficient formation of this intermediate requires Mu A, Mu B and E. coli HU proteins along with ATP and Mg^{++}. The Mu A protein binds to the Mu end DNA sequences on the donor DNA by one domain and to the internal Mu operator DNA site by another domain to form a special protein-DNA complex necessary to initiate the Mu DNA strand transfer reaction. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence to yield cleaved donor DNA with tightly associated Mu A proteins. This protein-DNA complex efficiently captures a second DNA molecule provided it is bound by Mu B protein. A staggered cut is introduced into the target DNA and each 5' end is joined to each of the 3' ends of the Mu end sequences by a concerted DNA cutting and joining reaction. Evidence has been obtained that this step of the reaction takes place by one step transesterification mechanism.

The Mu B protein, an ATPase, selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The Mu B protein dissociates selectively from the DNA molecule to which Mu A protein is bound in a process that depends on hydrolysis of ATP. Kinetic aspects of this energy transduction system are studied.

Studies of the Mechanism of Retroviral DNA Integration

Integration of a DNA copy of the retroviral genome into a chromosome of an infected cell is an essential step for normal viral replication. Our objectives are to analyze the detailed molecular mechanism of the integration reaction and facilitate the development of inhibitors of this step in the retroviral replication cycle.

We have demonstrated that a single protein (IN protein) encoded by HIV mediates the central steps of the integration reaction. This protein has been expressed in E. coli and purified in active form. HIV IN protein has a site-specific nuclease activity that cleaves two bases from each 3' end of the viral DNA and a DNA strand transfer activity that joins the resulting recessed 3' ends to the target DNA. A set of mutant HIV IN proteins has also been purified. Mutations that reduce the site-specific nuclease activity have a parallel effect on the strand transfer activity, suggesting that the active site(s) for the two reactions are intimately related. The mechanism of the HIV DNA strand transfer reaction has been investigated by carrying out in vitro reactions using a target DNA containing chiral phosphorothioate groups. The results support a single step transesterification mechanism.

Experiments to determine the extent of HIV terminal DNA sequence required for the cleavage and strand transfer reactions reveal that only a few nucleotide positions proximal to the very end of the viral DNA are critical for these reactions.

A simple in vitro assay system has been developed that is suitable for screening potential inhibitors of HIV DNA integration. Only short oligonucleotides matching an end of HIV DNA and purified HIV IN protein are required as substrates. Since each step of the assay can be carried out in the wells of microtiter plates, large numbers of reactions can be processed simultaneously.

Studies of Immunoglobulin Gene Rearrangement

The major purpose of this project is to dissect the mechanism by which immunoglobulin and T-cell receptor genes are assembled into functional entities, and to study the regulation of this reaction.

Several new results on this process, generally called V(D)J recombination, have been obtained.

(1) Recombinants formed in fibroblast cell lines which express the RAG-1 and RAG-2 genes rarely contain the added nucleotides which are characteristic of recombinants from lymphoid cells. The probable explanation is that fibroblasts lack the enzyme terminal deoxynucleotidyl transferase.

(2) The recombinational defect of cell lines derived from mice with the scid mutation can be partly overcome by altering the cell culture conditions. The mutation is therefore leaky.

(3) A molecular explanation for the preference of deletional over inversional D to J recombination at the immunoglobulin heavy-chain locus has emerged. The difference is due to several individually minor mutations in the upstream signal sequence associated with D regions.

Studies of Functions Involved in Genetic Recombination

The present focus of this project is to understand how DNA supercoiling is brought about by DNA gyrase and related enzymes, and to study the effects of DNA supercoiling on bacterial gene expression. These are some recent results:

The extremely slow binding of the ATP analog (β, γ-imido) ATP to DNA gyrase is greatly accelerated in the presence of ATP. Thus paradoxically ATP can help the analog to block ATP binding sites and inhibit the enzyme.

The effect of DNA supercoiling on bacterial transcription has been further studied. In a promoter that is induced by DNA relaxation, the structure of the first transcribed region turns out to be important; when the DNA is supercoiled, many transcripts terminate prematurely, while full-length transcripts are formed on relaxed DNA.

Experiments on the reverse DNA gyrase of thermophilic bacteria have shown that, unlike all other topoisomerases tested, this enzyme can only change supercoiling in one direction (toward positive supercoiling). Reverse gyrase is ATP-dependent, but can function with a remarkably low concentration of ATP.

Nonheritable Antibiotic Resistance

Growth in salicylate (SAL) increases the resistance of a number of Gram-negative bacteria to a variety of clinically important antibiotics. We have traced this effect in Escherichia coli to a reduction in the permeability of the outer membrane due to sharply reduced synthesis of OmpF, a major channel-forming outer membrane protein (porin). The transcription and translation of ompF and other relevant genes was studied by means of lacZ fusions, SDS gel electrophoresis of outer membrane proteins, and by measurements of outer membrane permeation. Growth in broth containing different concentrations of SAL reduced ompF-specific translation by as much as 99% (at 10 mM). However, ompF transcription was not affected by SAL. SAL decreases ompF translation in two ways. The first involves increasing micF transcription. Since micF transcripts are anti-sense to the 5' region of ompF transcripts, they decrease the translation of ompF mRNA by hybridizing with it. SAL increased micF transcription by 3- to 4-fold. In the absence of micF, the effect of SAL on ompF translation was clearly diminished, showing that it plays an important role in decreasing the amounts of OmpF in the outer membrane. However, since a considerable effect of SAL on ompF translation was evident even in the absence of micF, we infer the existence of a second (unknown) mechanism that reduces ompF translation in response to SAL.

In addition to affecting on ompF expression, growth in SAL increased the transcription of ompC, the gene for a second major porin. This was particularly obvious in the case of certain regulatory mutants that have very low levels of ompC transcription when grown without SAL. Since micF and ompC are divergently transcribed from a sequence that lies between them, we have proposed that growth in SAL specifically facilitates the joint transcription of micF and ompC.

Channeling in the Biosynthesis of Histidine

Channeling (preferential use of intermediates bound to enzyme complexes) has been proposed in several metabolic pathways. In systems where it operates, it could increase the overall catalytic efficiency.

No evidence could be found for channeling of substrates in the biosynthetic pathway of histidine. Radioactive imidazole glycerol phosphate (IGP) was synthesized chemically and its composition verified by a number of criteria. An electrophoretic assay for separating each of the intermediates in histidine biosynthesis from IGP to histidine was developed. Extracts were prepared from bacteria containing high levels of the enzymes and the kinetics of disappearance of IGP and the appearance of the intermediate and products were carefully monitored. Even in the presence of polyethylene glycol to provide crowded conditions, no channeling of IGP to histidine was observed.

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

The telomeric DNA sequences $(TTTGGG)_n$ and $(TTAGGG)_n$ where n = 12 and 24 form ordered structures which show large variations in stability with the nature of the alkali metal counterion, as do the CD spectra of the complexes. Concentration dependence of T_m suggests that for n = 12 the complexes are intermolecular, whereas for n = 24 they are composed of G quartets with intramolecular loops.

Hoogsteen base pairing at N7 of purines is known in the third strand of triple helices. We have now obtained "unconstrained" Hoogsteen TC·AG double helices with parallel strands. The only Hoogsteen duplexes reported previously were those we had prepared by use of blocking groups at C2 of A to force use of the alternative binding site. The duplexes exhibit strong inverse dependence of T_m on pH because of the need to protonate C for binding to N7 of G.

The triple helix poly d(A)·2poly d(T) was assigned the A conformation by Arnott and Selsing. We have now observed in the infrared spectrum in D_2O solution a band at 838 cm^{-1} which is characteristic of B form DNA and no A form band at 810^{-1}. We conclude that the helix has the B and not the A conformation and from modeling studies that the third strand has the reverse Hoogsteen structure and is antiparallel to the A strand. Similar conclusions were reached for the triple helix poly d(C-T) poly d(A-G)·poly dC^+-T).

AG and AI mispairs introduced in dGGTACGCGTACC cause little change in the UV thermal transition in the central 6,7 position but a large depression when moved out one position to 5,8. Further movement to the 4,9 and 3,10 positions results in a return of T_m to approximately that of the basic sequence. The anomalously low value for 5,8 AG or AI is due to formation of a four-base loop rather than a two-base loop for the 6,7 mispairs of the basic sequence. The cause of the return of T_m values to "baseline" is not yet clear, but it is possible that duplexes with single base loops rather than hairpinned monomers may be responsible.

Thermal Measurements of Biomolecular Systems

Using a highly sensitive pulsed-flow microcalorimeter, we have determined the changes in enthalpy and heat capacity for Cro repressor protein-DNA association reactions. The effect of base pair substitutions in the operator region of synthetic oligomeric (21 bp) DNAs upon the thermodynamics of reaction with a mutant (Val 27) and wild type Cro was investigated.

(1) The ΔH accompanying formation of the specific Cro repressor-operator OR3 DNA complex is endothermic below 18°C and exothermic above 18°C. At 15°C, $\Delta C_p = -360$ cal $mol^{-1}deg^{-1}$.

(2) The ΔH upon formation of the nonspecific DNA complex is about +4 kcal mol^{-1} and ΔC_p is zero.

(3)ΔC_p values for the formation of specific complexes with intermediate affinities (in binding of DNA and protein mutants) fall in between. There is a strong correlation between $\Delta G°$ for specific binding ($= \Delta G°_{specific} - \Delta G°_{nonspecific}$) and heat capacity change given by $\Delta\Delta G°$(cal mol^{-1}) = 18 (deg) x $\Delta\Delta C_p$ (cal $mol^{-1}deg^{-1}$). No correlation is observed between other thermodynamic parameters.

Examination of the data for the reactions of Cro with base pair substitution mutants suggests that the differences in the measured values of the thermodynamic parameters derive from perturbations of specific hydrogen bonds and van der Waals' interactions within the complex rather than from differences in general interactions with the solvent environment.

Influences of Macromolecular Crowding on Biochemical Systems

The very high concentration of macromolecules within cells can potentially have an overwhelming effect on the thermodynamic activity of cellular components because of excluded volume effects. To estimate the magnitudes of these effects, we have made an experimental study of the cytoplasm of Escherichia coli. Parameters from cells and cell extracts are used to calculate approximate activity coefficients for cytoplasmic conditions. These calculations require a representation of the sizes, concentrations, and effective specific volumes of the macromolecules in the extracts. Macromolecule size representations are obtained from two-phase partition and gel filtration assays. Macromolecule concentrations in the cytoplasm of E.coli are obtained from experiments using the known impermeability of the cytoplasmic volume to sucrose. Macromolecule specific volumes are inferred from the composition of close-packed precipitates induced by polyethylene glycol.

Several well-characterized proteins which bind to DNA (lac repressor, RNA polymerase) are extremely sensitive to changes in salt concentration in in vitro studies, but are insensitive in in vivo studies. Application of the activity coefficients from the present work indicates that at least part of this discrepancy arises from the difference in excluded volumes in these studies.

Enzyme Structure

1) Several data sets of the tryptophan synthase crystals from Salmonella typhimurium have been refined. The refined structures have confirmed the original observation at lower resolution of a tunnel for the passage of indole generated in the first reaction to the active site of the second enzyme. Mutants

are being examined with a view to elucidating the dynamic nature of this tunnel and of the site interaction in the complex.

2) Inhibitor complexes of rhizopus pepsin have been analyzed by X-ray diffraction with a view to obtaining more information concerning the mechanism of action.

3) A method has been developed for the joint refinement of structures determined by X-ray diffraction and by NMR.

4) Crystals have been obtained for the complex of a Y-box protein from Xenopus oocytes with a DNA oligomer.

AIDS Related Proteins: Structure and Function

1) Crystals of the Rnase H from HIV have been investigated. The crystals diffract to about 3.5A resolution. Many heavy atom derivatives have been examined in order to provide an interpretable electron density map.

2) Crystals obtained of the OKT4A/CD4 complex have been grown to X-ray dimensions. Examination of these crystals showed that they do not diffract to atomic resolution. The crystallization conditions for the HIV integrase protein are being investigated.

Three-Dimensional Structures of Cytokines, Receptors and Immune System Proteins

1) The refinement of the Fab structure of McPC603 has been completed.

2) Similar procedures are being applied to the refinement of the structure of the Fab/antigen complex HyHEL-5.

3) The structure of the lactose specific lectin from erythrina has been analyzed to completion.

4) Progress has been made in the crystal structure analysis of the transforming growth factor TGFbeta.

5) Crystals have been obtained of the EGF receptor.

Structural Studies of Molecular Recognition

1) Crystals of TSST-1 of S. aureus, the major toxin in Toxic Shock Syndrome, have been obtained.

2) Crystals of the Fab of CC49, a murine monoclonal antibody against solid adenocarcinomas, have been obtained.

3) A possible procedure for the 'humanization' of xenogeneic antibodies has been developed as an alternative to CDR-grafting.

4) A model of the Fc of human IgE, that incorporates the recently deduced inter ε chain disulfide linkage, has been built.

5) The preferred reading frames in the expression of the D regions of immunoglobulin heavy chains and of the T-cell receptor β-chains have been analyzed and correlated with possible structural constraints.

Study of the Potential Use of Catalytic Antibodies Against AIDS

Several highly-conserved polypeptide segments from the HIV-1 envelope glycoprotein have been identified as potential substrates for a proteolytic antibody. Peptidic transition-state analogs derived from these sequences have been synthesized.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33000-25 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Studies of Functions Involved in Genetic Recombination

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Martin Gellert	Chief, Section on Metabolic Enzymes,	LMB/NIDDK
OTHERS:	James K. Tamura	Staff Fellow	LMB/NIDDK
	Sergei Kozyavkin	Visiting Associate	LMB/NIDDK
	Regis Krah	IRTA Fellow	LMB/NIDDK
	Mary H. O'Dea	Research Chemist	LMB/NIDDK

COOPERATING UNITS *(if any)*

Dr. Andrew Bates, U. of Leicester, Leicester, U.K.
Dr. Rolf Menzel, Bristol-Myers-Squibb, Princeton, N. J.

LAB/BRANCH Laboratory of Molecular Biology

SECTION Section on Metabolic Enzymes

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS 3.7	PROFESSIONAL: 3.7	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

The present focus of this project is to understand how DNA supercoiling is brought about by DNA gyrase and related enzymes, and to study the effects of DNA supercoiling on bacterial gene expression. These are some recent results:

The extremely slow binding of the ATP analog (β, γ-imido) ATP to DNA gyrase is greatly accelerated in the presence of ATP. Thus paradoxically ATP can help the analog to block ATP binding sites and inhibit the enzyme.

The effect of DNA supercoiling on bacterial transcription has been further studied. In a promoter that is induced by DNA relaxation, the structure of the first transcribed region turns out to be important; when the DNA is supercoiled, many transcripts terminate prematurely, while full-length transcripts are formed on relaxed DNA.

Experiments on the reverse DNA gyrase of thermophilic bacteria have shown that, unlike all other topoisomerases tested, this enzyme can only change supercoiling in one direction (toward positive supercoiling). Reverse gyrase is ATP-dependent, but can function with a remarkably low concentration of ATP.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33001-7 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Studies of Immunoglobulin Gene Rearrangement

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
OTHERS:	David Brown	Clinical Associate	LMB/NIDDK
	Joanne Hesse	Research Chemist	LMB/NIDDK
	Fraser McBlane	Visiting Fellow	LMB/NIDDK
	Joseph Menetski	Guest Researcher	LMB/NIDDK
	David Roth	Staff Fellow	LMB/NIDDK
	Moshe Sadofsky	Special Volunteer	LMB/NIDDK

COOPERATING UNITS (if any)

Dr. Susanna Lewis, California Inst. of Technology
Dr. Michael Lieber, Stanford University

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Laboratory of Molecular Biology

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Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.8	6.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major purpose of this project is to dissect the mechanism by which immunoglobulin and T-cell receptor genes are assembled into functional entities, and to study the regulation of this reaction.

Several new results on this process, generally called V(D)J recombination have been obtained.

(1) Recombinants formed in fibroblast cell lines which express the RAG-1 nd RAG-2 genes rarely contain the added nucleotides which are characteristic of recombinants from lymphoid cells. The probable explanation is that fibroblasts lack the enzyme terminal deoxynucleotidyl transferase.

(2) The recombinational defect of cell lines derived from mice with the scid mutation can be partly overcome by altering the cell culture conditions. The mutation is therefore leaky.

(3) A molecular explanation for the preference of deletional over inversional D to J recombination at the immunoglobulin heavy-chain locus has emerged. The difference is due to several individually minor mutations in the upstream signal sequence associated with D regions.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33006-13 LMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Mechanism of Genetic Recombination

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Kiyoshi Mizuuchi	Chief, Section on Genetic Mechanisms LMB/NIDDK	
Others:	K. Adzuma	Visiting Associate	LMB/NIDDK
	M. Mizuuchi	Visiting Associate	LMB/NIDDK
	T. Baker	Special Volunteer	LMB/NIDDK

COOPERATING UNITS *(if any)*

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Laboratory of Molecular Biology

SECTION
Section on Genetic Mechanisms

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.5
PROFESSIONAL: 3.5
OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major objective of this project is to uncover the enzymatic steps involved in various genetic rearrangement reactions and to study the mechanism of action of the enzymes involved. The mechanism of the transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate a branched DNA intermediate. Efficient formation of this intermediate requires Mu A, Mu B and _E. coli_ HU proteins along with ATP and Mg^{++}. The Mu A protein binds to the Mu end DNA sequences on the donor DNA by one domain and to the internal Mu operator DNA site by another domain to form a special protein-DNA complex necessary to initiate the Mu DNA strand transfer reaction. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence to yield cleaved donor DNA with tightly associated Mu A proteins. This protein-DNA complex efficiently captures a second DNA molecule provided it is bound by Mu B protein. A staggered cut is introduced into the target DNA and each 5' end is joined to each of the 3' ends of the Mu end sequences by a concerted DNA cutting and joining reaction. Evidence has been obtained that this step of the reaction takes place by one step transesterification mechanism.

The Mu B protein, an ATPase, selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The Mu B protein dissociates selectively from the DNA molecule to which Mu A protein is bound in a process that depends on hydrolysis of ATP. Kinetic aspects of this energy transduction system are studied.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34001-26 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chromatin Structure and Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Gary Felsenfeld, Chief, Section on Physical Chemistry LMB/NIDDK

OTHERS:

David Clark, Visiting Associate	LMB/NIDDK	Joseph Knezetic, Staff Fellow	LMB/NIDDK
Jay Chung, Commissioned Officer	LMB/NIDDK	Catherine Lewis, Senior Staff Fellow	LMB/NIDDK
Henryk Eisenberg, Visiting Scientist	LMB/NIDDK	Mark Minie, Staff Fellow	LMB/NIDDK
Todd Evans, Staff Fellow	LMB/NIDDK	Joanne Nickol, Research Chemist	LMB/NIDDK
Gretchen Gibney, Special Volunteer	LMB/NIDDK	Marc Reitman, Senior Staff Fellow	LMB/NIDDK
Hannah Gould, Expert	LMB/NIDDK	Cecelia Trainor, IRTA Fellow	LMB/NIDDK

COOPERATING UNITS *(if any)*

Department of Biophysics, King's College (Robert Hannon)
Nat'l Inst. of Child Health and Human Develop., NIH (Heiner Westphal)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10.5	10.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have cloned the gene for the general erythroid-specific factor GATA-1 (Eryf1) of chicken. We find that the promoter of the gene contains binding sites for a number of known trans-acting factors, including a cluster of sites for GATA-1 itself. Transactivation studies show that these sites function in an autoregulatory mechanism. Other features of the promoter suggest mechanisms that may function early in development. We have continued studying the stage-specific expression of globin genes. We find that the π-globin gene, an embryonic α-family gene, is controlled by a complex mechanism involving three factors that change their concentration at the embryonic-adult switch. We have continued studies of the effects of a β-globin locus control region (LCR or DCR) on gene expression and chromatin structure. The LCR confers position independence of expression on nearby genes. We find that it is also capable of generating a hypersensitive domain in the absence of other elements, while elements like the β-globin promoter are not, a distinction important in understanding how active chromatin is generated. In order to understand how the histones of chromatin function during transcription, we have also studied the effect of positive supercoiling on nucleosome core formation. We find that core particles formed on such DNA are perfectly normal, but spontaneously transfer to negatively supercoiled DNA when that is available. We have proposed a mechanism, based on these observations, for transcription through nucleosome-covered DNA.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 34002-27 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Enzyme Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	David R. Davies, Chief, Sect. on Molecular Structure	LMB/NIDDK
Others:	C.Craig Hyde, Senior Staff Fellow	LMB/NIDDK
	Kevin D. Parris, IRTA Fellow	LMB/NIDDK
	John R.H. Gilstad, Guest Researcher	LMB/NIDDK

COOPERATING UNITS *(if any)*

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Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	3.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Summary:

1) Several data sets of the tryptophan synthase crystals from Salmonella typhimurium have been refined. The refined structures have confirmed the original observation at lower resolution of a tunnel for the passage of indole generated in the first reaction to the active site of the second enzyme. Mutants are being examined with a view to elucidating the dynamic nature of this tunnel and of the site interaction in the complex.

2) Inhibitor complexes of rhizopus pepsin have been analyzed by X-ray diffraction with a view to obtaining more information concerning the mechanism of action.

3) A method has been developed for the joint refinement of structures determined by X-ray diffraction and by NMR.

4) Crystals have been obtained for the complex of a Y-box protein from Xenopus oocytes with a DNA oligomer.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34003-23 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Three-Dimensional Structures of Cytokines, Receptors and Immune System Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	David R. Davies, Chief, Sect. on Molecular Structure	LMB/NIDDK
Others:	Sun Daopin, Visiting Scientist	LMB/NIDDK
	Christina Brown, Visiting Associate	LMB/NIDDK
	Gerson H. Cohen, Research Chemist	LMB/NIDDK
	Enid W. Silverton, Research Chemist	LMB/NIDDK
	Boaz Shaanan, Visiting Scientist	LMB/NIDDK

COOPERATING UNITS *(if any)*

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Summary:

1) The refinement of the Fab structure of McPC603 has been completed.

2) Similar procedures are being applied to the refinement of the structure of the Fab/antigen complex HyHEL-5.

3) The structure of the lactose specific lectin from erythrina has been analyzed to completion.

4) Progress has been made in the crystal structure analysis of the transforming growth factor TGFbeta.

5) Crystals have been obtained of the EGF receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 35000-27 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. Todd Miles, Chief, Section on Organic Chemistry		LMB/NIDDK
Others:	F. B. Howard	Research Chemist	LMB/NIDDK
	J. Frazier	Research Chemist	LMB/NIDDK
	Keling Liu	Visiting Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Girjesh Govil TIFR, India / Philip Ross LMB/NIDDK
V. Sasisekharan Visiting Scientist; Indian Instit. of Science, Banglagore, India
N. Patibiraman Naval Research Laboratory, Washington, D. C.
C-Q Chen Biotechnology Center, Shanghai

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Laboratory of Molecular Biology

SECTION

Section on Organic Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 5	PROFESSIONAL: 5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The telomeric DNA sequences $(TTTGGG)_n$ and $(TTAGGG)_n$ where n = 12 and 24 form ordered structures which show large variations in stability with the nature of the alkali metal counterion, as do the CD spectra of the complexes. Concentration dependence of T_m suggests that for n = 12 the complexes are intermolecular, whereas for n = 24 they are composed of G quartets with intramolecular loops.

Hoogsteen base pairing at N7 of purines is known in the third strand of triple helices. We have now obtained "unconstrained" Hoogsteen TC·AG double helices with parallel strands. The only Hoogsteen duplexes reported previously were those we had prepared by use of blocking groups at C2 of A to force use of the alternative binding site. The duplexes exhibit strong inverse dependence of T_m on pH because of the need to protonate C for binding to N7 of G.

The triple helix poly d(A)·2poly d(T) was assigned the A conformation by Arnott and Selsing. We have now observed in the infrared spectrum in D_2O solution a band at 838 cm^{-1} which is characteristic of B form DNA and no A form band at 810^{-1}. We conclude that the helix has the B and not the A conformation and from modeling studies that the third strand has the reverse Hoogsteen structure and is antiparallel to the A strand. Similar conclusions were reached for the triple helix poly d(C-T) poly d(A-G)·poly dC^{+}-T).

AG and AI mispairs introduced in dGGTACGCGTACC cause little change in the UV thermal transition in the central 6,7 position but a large depression when moved out one position to 5,8. Further movement to the 4,9 and 3,10 positions results in a return of T_m to approximately that of the basic sequence. The anomalously low value for 5,8 AG or AI is due to formation of a four-base loop rather than a two-base loop for the 6,7 mispairs of the basic sequence. The cause of the return of T_m values to "baseline" is not yet clear, but it is possible that duplexes with single base loops rather than hairpinned monomers may be responsible.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 35050 20 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Replication, Recombination and Repair of Microbial DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Tomizawa	Visiting Scientist	LMB/NIDDK
OTHERS:	Y. Eguchi	Visiting Fellow	

COOPERATING UNITS *(if any)*

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Laboratory of Molecular Biology

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: PROFESSIONAL: OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project terminated in FY '91.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 36003-7 LMB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Nonheritable Antibiotic Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. L. Rosner	Research Biologist	LMB/NIDDK
Others:	J. D. Foulds	Research Chemist	LSB/NIDDK
	Reeta Goel	Visiting Associate	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Microbial Genetics

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS 2.25	PROFESSIONAL 2.25	OTHER 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

Growth in salicylate (SAL) increases the resistance of a number of Gram-negative bacteria to a variety of clinically important antibiotics. We have traced this effect in Escherichia coli to a reduction in the permeability of the outer membrane due to sharply reduced synthesis of OmpF, a major channel-forming outer membrane protein (porin). The transcription and translation of ompF and other relevant genes was studied by means of lacZ fusions, SDS gel electrophoresis of outer membrane proteins, and by measurements of outer membrane permeation. Growth in broth containing different concentrations of SAL reduced ompF-specific translation by as much as 99% (at 10 mM). However, ompF transcription was not affected by SAL. SAL decreases ompF translation in two ways. The first involves increasing micF transcription. Since micF transcripts are anti-sense to the 5' region of ompF transcripts, they decrease the translation of ompF mRNA by hybridizing with it. SAL increased micF transcription by 3- to 4-fold. In the absence of micF, the effect of SAL on ompF translation was clearly diminished, showing that it plays an important role in decreasing the amounts of OmpF in the outer membrane. However, since a considerable effect of SAL on ompF translation was evident even in the absence of micF, we infer the existence of a second (unknown) mechanism that reduces ompF translation in response to SAL.

In addition to affecting on ompF expression, growth in SAL increased the transcription of ompC, the gene for a second major porin. This was particularly obvious in the case of certain regulatory mutants that have very low levels of ompC transcription when grown without SAL. Since micF and ompC are divergently transcribed from a sequence that lies between them, we have proposed that growth in SAL specifically facilitates the joint transcription of micF and ompC.

PHS 6040 (Rev 1/84) U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 36051-23 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mammalian DNA Replication, Regulation and Amplification

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert G. Martin	Chief, Sec. on Microbial Genetics	LMB/NIDDK
Others:	B. S. Rao	Visiting Fellow	LMB/NIDDK
	M. Reitman	Senior Staff Fellow	LMB/NIDDK

COOPERATING UNITS *(if any)*

Foreign: M. Zannis-Hadjopoulos, McGill Cancer Center, Montreal, Canada
G. Kaufmann, Tel Aviv University, Tel Aviv, Israel
H. Manor, Technicon University, Haifa, Israel

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was terminated in FY 91.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 36101-17 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Energy Conversion in Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Yi-der Chen	Research Chemist	LMB/NIDDK
Others:	Robert J. Rubin	Special Volunteer	LMB/NIDDK

COOPERATING UNITS *(if any)*

Shahid Khan, Albert Einstein College of Medicine, NY, NY 10461
Aydin Tozeren, Catholic University, Washington, D. C. 20064

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Researchers transferred to Laboratory of Chemical Physics in October 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36102-20 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Statistical Thermodynamics of Protein and Polynucleotide Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Yi-der Chen	Research Chemist	LMB/NIDDK

COOPERATING UNITS *(if any)*

J. Chalovich, University East Carolina, North Carolina

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Laboratory of Molecular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Researcher transferred to Laboratory of Chemical Physics in October 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36104-10 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Thermal Measurements of Biomolecular Systems

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	P.D. Ross	Research Chemist	LMB/NIDDK
Others:	A.C. Steven	Visiting Scientist	LPB/NIAMS
	A. Shrake	Research Chemist	DBBP/CPB
	M.S. Lewis	Research Chemist	BEIP/NCRR
	Y. Takeda	Research Chemist	FCRF/NCI
	C.P. Mudd	Engineer	BEIP/RR

COOPERATING UNITS (*if any*)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2	PROFESSIONAL: 2	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Using a highly sensitive pulsed-flow microcalorimeter, we have determined the changes in enthalpy and heat capacity for Cro repressor protein-DNA association reactions. The effect of base pair substitutions in the operator region of synthetic oligomeric (21 bp) DNAs upon the thermodynamics of reaction with a mutant (Val 27) and wild type Cro was investigated.

(1) The ΔH accompanying formation of the specific Cro repressor-operator OR3 DNA complex is endothermic below 18°C and exothermic above 18°C. At 15°C, $\Delta C_p = -360$ cal $mol^{-1} deg^{-1}$.

(2) The ΔH upon formation of the nonspecific DNA complex is about +4 kcal mol^{-1} and ΔC_p is zero.

(3) ΔC_p values for the formation of specific complexes with intermediate affinities (in binding of DNA and protein mutants) fall in between. There is a strong correlation between ΔG° for specific binding ($= \Delta G^\circ_{specific} - \Delta G^\circ_{nonspecific}$) and heat capacity change given by $\Delta\Delta G^\circ$ (cal mol^{-1}) = 18 (deg) x $\Delta\Delta C_p$ (cal $mol^{-1} deg^{-1}$). No correlation is observed between other thermodynamic parameters.

Examination of the data for the reactions of Cro with base pair substitution mutants suggests that the differences in the measured values of the thermodynamic parameters derive from perturbations of specific hydrogen bonds and van der Waals' interactions within the complex rather than from differences in general interactions with the solvent environment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36105-09 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Influences of Macromolecular Crowding on Biochemical Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S.B. Zimmerman, Research Chemist LMB/NIDDK

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The very high concentration of macromolecules within cells can potentially have an overwhelming effect on the thermodynamic activity of cellular components because of excluded volume effects. To estimate the magnitudes of these effects, we have made an experimental study of the cytoplasm of Escherichia coli. Parameters from cells and cell extracts are used to calculate approximate activity coefficients for cytoplasmic conditions. These calculations require a representation of the sizes, concentrations, and effective specific volumes of the macromolecules in the extracts. Macromolecule size representations are obtained from two-phase partition and gel filtration assays. Macromolecule concentrations in the cytoplasm of E.coli are obtained from experiments using the known impermeability of the cytoplasmic volume to sucrose. Macromolecule specific volumes are inferred from the composition of close-packed precipitates induced by polyethylene glycol.

Several well-characterized proteins which bind to DNA (lac repressor, RNA polymerase) are extremely sensitive to changes in salt concentration in in vitro studies, but are insensitive in in vivo studies. Application of the activity coefficients from the present work indicates that at least part of this discrepancy arises from the difference in excluded volumes in these studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36106-04 LMB

PERIOD COVERED

October 1, 1990 to May 19, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Developmental Regulation of Differential Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alan Wolffe, Visiting Scientist LMB/NIDDK

OTHERS:

Constantin Chipev, Visiting Associate	LMB/NIDDK	
Mary Familari, Visiting Fellow	LMB/NIDDK	
Jeffrey Hayes, Special Volunteer	LMB/NIDDK	(NRC Fellow)
Sherrie Tafuri, Special Volunteer	LMB/NIDDK	(NRC Fellow)

COOPERATING UNITS (if any)

Dept. Chem. Johns Hopkins Univ. (T. Tullius, J. Bashkin); Lab. of Mol. Carcinogenesis NCI (M. Bustin, M. Crippa); Inst. Jacques Monod, Paris, France (M. Mechali, G. Almouzni); Inst. Biologie Animale Lausanne Switzerland (W. Wahli, C. Schild); Lab. of Cell and Develp. Biol. NIDDK (R. Morse); Dept. Biochem. Southwestern Med. Centre San Antonio (J. Hansen)

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Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	2.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our work is focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogenesis. Progress has been achieved in three key areas:

1. We have defined at single base pair resolution the protein-nucleic acid interactions over active and inactive 5S ribosomal RNA genes in vivo. These results strongly support a role for stable transcription complexes in maintaining gene activity and for nucleosomes in maintaining the repressed state.

2. We have defined the structure of DNA in a nucleosome and the contributions of histone domains to that structure. We find that DNA sequences of widely divergent structure in solution adopt the same structure in the nucleosome. Purified transcription factors interact with comparable affinity to their promoters as naked DNA and when associated with histones H3/H4. Histone H2A/H2B, when incorporated into a nucleosome inhibit transcription factor binding. These results provide a molecular explanation for the access of transcription factors to DNA during staged chromatin assembly at the replication fork.

3. We have defined a new DNA binding motif in the Y-box transcription factors. Germ cell selective variants of these factors have been cloned from Xenopus and mouse respectively suggesting a developmental role that has been conserved through evolution.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36108-04 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Studies of the Mechanism of Retroviral DNA Integration

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Robert Craigie	Visiting Scientist	LMB/NIDDK
	Kiyoshi Mizuuchi	Chief, Genetic Mechanisms	LMB/NIDDK
Others:	Frederic Bushman	Special Volunteer	LMB/NIDDK
	Alan Engelman	IRTA	LMB/NIDDK
	Myung-Soo Lee	Special Volunteer	LMB/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 4	PROFESSIONAL: 4	OTHER 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Integration of a DNA copy of the retroviral genome into a chromosome of an infected cell is an essential step for normal viral replication. Our objectives are to analyze the detailed molecular mechanism of the integration reaction and facilitate the development of inhibitors of this step in the retroviral replication cycle.

We have demonstrated that a single protein (IN protein) encoded by HIV mediates the central steps of the integration reaction. This protein has been expressed in E. coli and purified in active form. HIV IN protein has a site-specific nuclease activity that cleaves two bases from each 3′ end of the viral DNA and a DNA strand transfer activity that joins the resulting recessed 3′ ends to the target DNA. A set of mutant HIV IN proteins has also been purified. Mutations that reduce the site-specific nuclease activity have a parallel effect on the strand transfer activity, suggesting that the active site(s) for the two reactions are intimately related. The mechanism of the HIV DNA strand transfer reaction has been investigated by carrying out in vitro reactions using a target DNA containing chiral phosphorothioate groups. The results support a single step transesterification mechanism.

Experiments to determine the extent of HIV terminal DNA sequence required for the cleavage and strand transfer reactions reveal that only a few nucleotide positions proximal to the very end of the viral DNA are critical for these reactions.

A simple in vitro assay system has been developed that is suitable for screening potential inhibitors of HIV DNA integration. Only short oligonucleotides matching an end of HIV DNA and purified HIV IN protein are required as substrates. Since each step of the assay can be carried out in the wells of microtiter plates, large numbers of reactions can be processed simultaneously.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36109-4 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

AIDS Related Proteins: Structure and Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	David R. Davies, Chief, Section on Molecular Structure	LMB/NIDDK
Other:	Boaz Shaanan, Visiting Scientist	LMB/NIDDK
	Enid W. Silverton, Research Chemist	LMB/NIDDK
	Christina J. Brown, Visiting Fellow	LMB/NIDDK
	Lucie Yang, Summer Student	LMB/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Summary:

1) Crystals of the RNase H from HIV have been investigated. The crystals diffract to about 3.5A resolution. Many heavy atom derivatives have been examined in order to provide an interpretable electron density map.

2) Crystals obtained of the OKT4A/CD4 complex have been grown to X-ray dimensions. Examination of these crystals showed that they do not diffract to atomic resolution. The crystallization conditions for the HIV integrase protein are being investigated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36110-03 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Gene Expression during Chicken Erythrocyte Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joanne Nickol, Research Chemist LMB/NIDDK

COOPERATING UNITS (if any)

Lab. of Molecular Carcinogenesis, NCI, NIH (M. Crippa, M. Bustin)

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Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.50	.50	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Developmental regulation of expression of two major gene families was studied: globin genes and HMG 14(a and b) and 17 genes. The former represents a differentiated function restricted to erythrocytes whereas the latter genes are constitutively expressed as an essential component of cellular chromatin.

A. Globin genes. Expression of the ϵ globin gene is restricted to the primitive erythrocyte, which circulates only early in chick embryogenesis (day 1-7). The promoter has been sequenced and putative control regions are being analyzed by in vitro footprooting and gel mobility shift assays. The extracts used for these analyses are derived from erythrocytes obtained at different stages of development spanning the time when the ϵ gene is fully active to the adult chicken, when all genes are totally inactive. These regions of differential binding within the ϵ promoter are also being analyzed for functional activity by linkage to a reporter gene and subsequent transfection into primary erythrocytes. A special β-globin reporter gene has been constructed for this purpose.

B. HMG 14(a and b) and 17. To establish this system we investigated various parameters related to expression of these genes in erythrocytes during embryogenesis: pulse-labeled protein, steady state mRNA, nuclear run-ons that analyze only newly synthesized transcripts, and chromatin structure of the genes themselves. Our results suggest that the individual genes of the HMG family are differentially expressed in development. HMG 14a, in particular, is restricted to the primitive (early) erythrocyte whereas HMG 14b and 17 are maximally expressed only later, in the definitive erythrocyte, but not in the reticulocyte.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36111-03 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural Molecular Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Joanne Nickol, Research Chemist LMB/NIDDK

COOPERATING UNITS *(if any)*

Laboratory of Biochemical Metabolism, NIDDK, NIH (D.C. Rau)
University of Calgary Medical School, Calgary, Alberta, Canada (D. Bazett-Jones)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.25	.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The position and magnitude of the Zn induced bend within the 5s RNA gene of Xenopus has been determined. The center of the bend is located at about bp +65 within the gene. The bend magnitude is at least 50-60 degrees, comparable to naturally occuring bends from phased A tracts, until now the only other sequence known to bend DNA. These values were obtained by quantitative analysis of changes in rotational diffusion coefficients with and without added Zn for a series of overlapping DNA fragments spanning the 5s RNA gene. Given the specificity of Zn binding for purines, the most probable cause of the bending is due to junction angles between oligopurines tracts with a Zn induced altered conformation and adjacent canonical B-form structures. Both the oocyte and somatic sequences have four of these oligopurine tracts within a 60 bp region. They are symmetrically located about a bp +70 center and have an underlying 10 bp phasing. This bend is found within the binding domain of TF IIIA, a zinc finger protein that plays a key role in regulating 5s RNA gene activity. There is evidence in the literature, as well as our own preliminary results, that TF IIIA binding also bends DNA. The binding strength of TF IIIA to sequence variants of the binding domains seems to correlate with the symmetry and phasing of the oligopurine tracts. The bending of DNA by TF IIIA binding is likely accomplished by stabilizing the same alternate DNA conformation as Zn binding.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36112-3 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of a Gene Expressed in Undifferentiated Teratorcacinoma Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Persico Visiting Scientist LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project was terminated in FY 91.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36113-2 LMB

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Channeling in the Biosynthesis of Histidine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Robert G. Martin Chief, Sec. on Microbial Genetics, LMB/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Molecular Biology

SECTION Section on Microbial Genetics

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
.50	.50	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Channeling (preferential use of intermediates bound to enzyme complexes) has been proposed in several metabolic pathways. In systems where it operates, it could increase the overall catalytic efficiency.

No evidence could be found for channeling of substrates in the biosynthetic pathway of histidine. Radioactive imidazole glycerol phosphate (IGP) was synthesized chemically and its composition verified by a number of criteria. An electrophoretic assay for separating each of the intermediates in histidine biosynthesis from IGP to histidine was developed. Extracts were prepared from bacteria containing high levels of the enzymes and the kinetics of disappearance of IGP and the appearance of the intermediate and products were carefully monitored. Even in the presence of polyethylene glycol to provide crowded conditions, no channeling of IGP to histidine was observed.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36114-1 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Structural Studies of Molecular Recognition

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eduardo A. Padlan, Ph.D.,	Visiting Scientist	LMB/NIDDK
Others:	Chantal Abergel, Ph.D.,	Visiting Fellow	LMB/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

SUMMARY:

1) Crystals of TSST-1 of *S. aureus*, the major toxin in Toxic Shock Syndrome, have been obtained.

2) Crystals of the Fab of CC49, a murine monoclonal antibody against solid adenocarcinomas, have been obtained.

3) A possible procedure for the 'humanization' of xenogeneic antibodies has been developed as an alternative to CDR-grafting.

4) A model of the Fc of human IgE, that incorporates the recently deduced inter ε chain disulfide linkage, has been built.

5) The preferred reading frames in the expression of the D regions of immunoglobulin heavy chains and of the T-cell receptor β-chains have been analyzed and correlated with possible structural constraints.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36115-1 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Study of the Potential Use of Catalytic Antibodies Against AIDS

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eduardo A. Padlan, Ph.D.,	Visiting Scientist	LMB/NIDDK

COOPERATING UNITS *(if any)*

COLLABORATORS:	ETTORE APPELLA, M.D.	MEDICAL OFFICER	LCB/NCI
	BIRGIT A. HELM, Ph.D.	LECTURER	UNIV. OF SHEFFIELD
	THOMAS J. KINDT, Ph.D.	CHIEF	LIG/NIAID

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

SUMMARY:

Several highly-conserved polypeptide segments from the HIV-1 envelope glycoprotein have been identified as potential substrates for a proteolytic antibody. Peptidic transition-state analogs derived from these sequences have been synthesized.

ANNUAL REPORT OF THE METABOLIC DISEASES BRANCH
National Institute of Diabetes and
Digestive and Kidney Diseases

The general goals of the Branch are to investigate the mechanism of action of hormones controlling ion transport and mineral metabolism and to investigate the immunological and pathological factors mediating kidney disorders. The branch currently includes sections of Mineral Metabolism (Dr. Marx), Endocrine Regulation (Dr. Aurbach), Renal Cell Biology (Dr. Striker) and Kidney Disease (Dr. Balow). Integration of these sections is related to common interests in the pathophysiology of metabolic disorders which interface with the kidney. Systems under study include renal and skeletal tissue, transgenic mice, isolated cells (kidney and parathyroid in culture), hormone receptors (beta adrenergic, parathyroid hormone, prostaglandin, adenosine calcitonin and 1,25 dihydroxy vitamin D), parathyroid cell growth factors, and T cell and B cell function in disorders of immunoregulation.

Analysis of Hormone Receptor

Interactions with several hormone receptors regulating growth or adenylate cyclase are under study. Specific receptors have now been identified on parathyroid cells, pineal cells, rat, guinea, pig, and monkey lung membrane preparations, bovine parathyroid endothelial cells, rat osteosarcoma cells, osteoclast-like cells and rat liver membranes. Control of receptor biosynthesis in isolated cell culture systems is being studied with a view toward gaining knowledge about the molecular biology of receptors and how they are linked to intracellular response systems. A new method has been developed for immunocytology of cAMP and cGMP to study compartmentalization of signal transduction.

Primary Hyperparathyroidism and Familial Hypercalcemia

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case finding studies have produced approximately 85 kindreds for analysis. These studies allowed segregation of the most common familial variants into two distinct disease syndromes - familial multiple endocrine neoplasia type 1 (FMEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from FMEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy.

Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. FHH accounts for approximately 10% of all unsuccessful parathyroidectomies in hypercalcemia. In several FHH kindreds one or more members have exhibited life-threatening primary hyperparathyroidism in the neonatal period. This may result sometimes from a double dose of the FHH gene. Dispersed parathyroid cells from one severely affected neonate showed a striking decrease in sensitivity of PTH secretion to extracellular calcium. This disorder may reflect mutation in a gene that directs calcium recognition in both the parathyroid and renal tubular cell

Familial multiple endocrine neoplasia type I (FMENI) is an autosomal dominant disorder characterized by hyperfunction of parathyroids, pancreatic islets, and anterior pituitary. Affected organs show features suggestive of increased proliferation. Virtually all subjects expressing the gene show primary hyperparathyroidism. Primary hyperparathyroidism is usually first recognizable between ages 20-40, and it shows a high recurrence rate after subtotal parathyroidectomy (approximately 50% after 10 years). We have evaluated multiple indices for use in screening in a very large kindred. We tested 221 members and newly identified 16 as carriers. Albumin-adjusted calcium and PTH were most useful; gastrin and prolactin analyses were not useful for screening but showed promise in followup of known carriers. Analysis in this family has revealed linkage to a locus on the long arm of chromosome 11. The MEN1 gene is likely a growth suppressor gene like the retinoblastoma gene; MEN1 related tumors are being screened for loss of heterozygosity at this locus. Such loss of heterozygosity has established that most parathyroid tumors in FMEN1 are monoclonal. Tumors with small deletions could speed identification of the MENI gene. Further studies suggest that inactivation of the MEN1 gene also contributes to neoplasia in over 25% of sporadic parathyroid adenomas. [Drs. Friedman, DeMarco, A. Bale, Sakaguchi, Brandi, Norton, Spiegel, Aurbach, Marx]

With cultured bovine parathyroid cells, we found abnormally high mitogenic activity in plasma from 23 of 27 subjects with FMEN1. The mitogenic factor(s) appears to be a protein of 14,000 mw. We have begun purifying this factor for further characterization. We have obtained immunological evidence that the factor is related to if not identical to basic fibroblast growth factor. Analysis of plasmas from one large kindred with FMENI suggests that high parathyroid mitogenic activity precedes primary hyperparathyroidism and may begin at very early ages. [Drs. Brandi, Sakaguchi, Aurbach, Marx].

Studies on noninvasive and invasive modes of localizing parathyroid tumors continue. Parathyroid adenoma localization has been evaluated using the new non-invasive magnetic resonance imaging technique. Initial results were disappointing but the acquisition of a specialized neck collar has led to better resolution in the paratracheal and mediastinal areas. Patients are currently under evaluation with this new technique. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 450 of cases tested, the identification of abnormal masses of tissue proven at surgery to be parathyroid. In the most difficult cases, localization of parathyroid tissue can be aided by identifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Fine needle aspiration is another new method that can obviate other invasive localization procedures. We have aspirated with guidance by computerized tomography or ultrasound approximately 20 such lesions that were subsequently confirmed surgically as parathyroid. RIA of the aspirates showed high concentrations of PTH in all but one. Eight mediastinal adenomas have been treated nonsurgically by percutaneous injection via catheter of occlusive agents into the arterial blood supply with 7 complete and one partial remissions. [Drs. Aurbach, Marx, Spiegel, Weinstein, Merendino, NIDDK: Dr. Norton, NCI, Drs. Doppman, Miller, and others, Diagnostic Radiology, CC].

Rapid determination of intraoperative UcAMP excretion (using the Gammaflo machine for rapid cAMP radioimmunoassay) has proven to be a valuable tool in guiding surgery for primary hyperparathyroidism, particularly in patients with multigland disease. Persistent elevation of UcAMP requires continued search for abnormal tissue even after 1 or more abnormal glands have been removed. A rapid (mean 1.5 hours) drop in UcAMP to less than 50% of the baseline obviates the need for continued exploration even in cases where histologic confirmation of parathyroidectomy is lacking. Spurts in UcAMP above baseline may provide a clue to the location of abnormal parathyroid tissue. [Drs. Spiegel, Marx, Merendino, Weinstein, and Aurbach, NIDDK: Dr. Norton, NCI Surgery].

Secretion of Parathyroid Hormone

PTH secretion from parathyroid glands *in vivo* and cells *in vitro* is controlled by intracellular calcium and cyclic AMP. Control by calcium is altered in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Agents that alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP, but most of its effect to inhibit secretion is independent of changes in cellular cAMP.

Calcium inhibition of parathyroid hormone secretion is controlled through a complex set of mechanisms. We have shown previously that classical voltage-sensitive calcium channels are important in controlling parathyroid hormone secretion and that their action is mediated through a guanine nucleotide regulatory protein. It is also known that the growth of parathyroid cells is controlled by calcium. We have utilized a cloned rat parathyroid cell line (PT-r) to study these phenomena.

Proteoglycans synthesized by the PT-r cell have been characterized and effects of calcium on synthesis studied. One proteoglycan is located both intracellularly and on the external surface of the cell. Distribution of this proteoglycan between cell cytosol and cell surface is controlled by calcium. This effect on cell surface proteoglycans may be important in calcium regulation of growth as well as secretion. [Drs. Sakaguchi, Yanagishita, Brandi, and Aurbach].

We have recently shown that the PT-r cell produces acidic FGF (aFGF) and also bears receptors for aFGF. Further studies show that aFGF receptors exist on the PT-r cell in high and low affinity forms. The higher affinity form proved to be a proteoglycan. Calcium controls release of aFGF from the cell and also regulates synthesis of the aFGF receptor. These mechanisms may be important mediators of calcium-controlled growth of the cell [Drs. Sakaguchi, Brandi, and Aurbach].

Endothelial cells have been cloned from bovine parathyroid tissue. These cells are distinct from the parathyroid epithelial cells that secrete hormone. As is characteristic of endothelial cells they contain Factor VIII-related antigen, take up acetylated low density lipoprotein and show ultrastructural features comparable to other endothelial cells. Using this cloned cell system we have shown that it is the target of antibodies developed in autoimmune hypoparathyroidism and also is the target for a parathyroid cell growth factor identified in multiple endocrine neoplasia type I. Classical H_2 type histamine receptors have been identified by function (cAMP production) and ligand binding studies in these cells. [Drs. Brandi, Sakaguchi, and Aurbach].

Vitamin D Resistance and Related Disorders

The role of $1,25(OH)_2D_3$, the most potent natural metabolite of vitamin D, has been assessed in hypocalcemic states. This very rapidly acting drug has simplified the management of hypocalcemia following parathyroidectomy: during this time skeletal remineralization imposes large but rapidly diminishing requirements for calcium.

We have evaluated patients with extreme resistance to $1,25(OH)_2D$. This can be a transient state as following parathyroidectomy or a permanent state as in familial cases. We have evaluated 20 patients with familial resistance to $1,25(OH)_2D$. Most patients display hypocalcemic rickets, usually with total alopecia. Alopecia is found among the highest grades of resistance to $1,25(OH)_2D$, implicating calcitriol in physiology of the hair follicle. Mineral homeostasis is usually improved by treatments that sustain $1,25(OH)_2D$ levels at 10-100 times normal.

Intestinal response to 1,25$(OH)_2$D can be documented repeatedly with a new technique utilizing a stable isotope of calcium [Drs. Yergey, Viera, Marx].

Specific intracellular defects have been evaluated using cultured skin fibroblasts from these patients. With skin fibroblasts cultured from normals, properties of the 1,25$(OH)_2$D-receptor can be identified by binding in soluble extracts, by nuclear uptake of hormone with intact cells, or by elution of occupied receptor from DNA-cellulose. Fibroblasts from patients with familial resistance to 1,25$(OH)_2$ have shown a spectrum of defects including nonfunctional receptors, diminished numbers of receptors, and receptors with decreased hormone binding affinity. Among cases displaying apparently normal hormone binding sites, some show receptors with deficient binding to nucleus while others show normal binding of hormone-receptor complex to the nucleus but abnormal interaction with nonspecific DNA (as DNA-cellulose). Cellular action of 1,25$(OH)_2D_3$ can be analyzed by measuring its induction of the 25(OH)D 24-hydroxylase enzyme system. Cultured skin fibroblasts from all patients with hereditary resistance to 1,25$(OH)_2$D exhibit defects in this induction. Immunocytology reveals multiple rapid steps of reorganization of vitamin D receptors after calcitriol addition. Specific disruptions in these steps can be imaged in mutant cells from patients. [Drs. Marx, Barsony, MDB, NIDDK; Dr. Liberman, Israel; Drs. Pike (Baylor) and DeLuca (Madison).

Fibroblast lines from patients with hereditary extreme resistance to 1,25$(OH)_2D_3$ are being used to probe for normal functions of the 1,25$(OH)_2D_3$ receptor. We have shown that 1,25$(OH)_2D_3$ can elevate intracellular cyclic GMP very rapidly (within 1-3 minutes). This response showed affinity and analog specificity characteristic of the 1,25$(OH)_2D_3$ receptor and was absent in all "mutant" fibroblast lines which, nevertheless, retained rapid cGMP responses to nitroprusside and to androgens. Thus a 1,25$(OH)_2D_3$ receptor mediates this rapid response. Immunocytology revealed that, after 1,25$(OH)_2D_3$ addition, cGMP accumulates rapidly about the reorganizing vitamin D receptors [Drs. Barsony, Marx].

KIDNEY DISEASE SECTION

Research focusses on human glomerular diseases, experimental models of immune-mediated renal disorders, and normal biology of various types of kidney cells. Patient and animal tissues are used to study pathogenetic mechanisms, primarily involving cytokines and growth factors. Mechanisms of action of immunosuppressive drugs at the level of gene regulation and transcription and novel immunosuppressive drug therapies which might have salutary effects on the course of lupus nephritis and membranous nephropathy are under study.

I. Glomerulonephritis and lupus nephritis

A. Immunopathogenesis. Murine models are being utilized to investigate the different components of lupus nephritis. The modulating effects of cyclophosphamide on immune responses in normal mice and on the renal lesions of nephritic mice are being investigated. Studies of differences among the murine strains have provided new approaches to study of the diverse manifestations and response to treatment of human lupus nephritis. (Austin, Patel, Balow).

B. Regulation of lymphocyte gene expression. Dysregulated cell mediated immune responses are present in subjects with most forms of glomerulonephritis. Studies of the mechanisms of control of B and T cell activation, including regulation of immunoglobulin and cytokine genes by nuclear factors are being pursued. Special emphasis is placed on studies of the effects of various immunoregulatory agents, such as corticosteroids, cyclosporine, and cyclophosphamide. (Boumpas, Paliogianni, Ahuja, Balow).

C. Proliferative lupus nephritis. Studies have shown that intermittent pulse cyclophosphamide therapy is superior to conventional prednisone in management of lupus nephritis. No direct comparisons of pulse corticosteroids and pulse cyclophosphamide have been performed. Patients with proliferative lupus nephritis are being treated with pulse methylprednisolone or pulse cyclophosphamide to compare these two types of drugs and to assess whether intensity or duration of cyclophosphamide therapy is more important in stabilizing the renal disease. Results to date indicate a significantly higher probability of patients developing progressive renal failure with methylprednisolone therapy compared to intermittent pulse cyclophosphamide. Immunologic studies of changes in lymphoid cell function by the various drug regimens are being pursued to identify techniques which will maximize efficacy and to improve monitoring of drug treatment. (Balow, Austin, Boumpas, MacKay and members of ARB, NIAMS).

D. Membranous nephropathy. Membranous nephropathy is associated with substantial cardiovascular morbidity from nephrotic syndrome and causes an insidious loss of renal function in patients with lupus and in those patients with idiopathic forms of this disease. Preliminary evidence indicates that the immunopathogenesis of membranous nephropathy is distinct from that of most proliferative forms of glomerulonephritis. Current protocols involve examination of the pathophysiology and histopathology of the glomerular lesions in membranous nephropathy, as well as evaluation of the comparative efficacy of prednisone, cyclophosphamide and cyclosporin A in patients idiopathic and lupus related forms of this renal disease. (Balow, Austin, MacKay).

E. Transforming growth factor and glomerular reactions. The mechanisms responsible for normal growth and for pathogenic cellular reactions within the glomerulus are poorly understood. The signal transducing agent, transforming growth factor-beta, has a complex interaction with glomerular cells. Studies are underway to characterize the nature of the receptors for this growth factor on glomerular cells. In addition, several parameters, such as proliferation, fibronectin secretion and proteoglycan synthesis, will be used to evaluate the responses of these cells to binding of transforming growth factor. (MacKay).

II. Role of Complement in Glomerulonephritis.

A. Nephritic factors. Patients with membranoproliferative glomerulonephritis and lupus nephritis develop autoantibodies to complement converting enzymes which leads to abnormal consumption of complement components. Anti-idiotypic antibodies to nephritic factors have been isolated. The characteristics of ligand binding, receptor turnover and modulation by the nephritic factors, and idiotypic binding sites are under study. (Tsokos).

B. Complement in immune regulation. Abnormal levels of complement components and deposition in sites of immunological reactions are characteristic of several forms of nephritis. The interactions of complement components and activation products with receptors on lymphoid cells are being studied to gain new insights into their potential role in lupus nephritis, membranoproliferative glomerulonephritis and other renal disorders. (Tsokos, Balow).

Studies of the pathogenesis of glomerulosclerosis

The renal cell biology section is investigating the cellular and molecular mechanisms leading to glomerular scarring (glomerulosclerosis), the emphasis being on non-immunologically mediated diseases. The major disease process of interest is the kidney disease of diabetes mellitus. Our general hypothesis is that abnormalities in the growth regulation of resident glomerular cells play a major role in the development of glomerulosclerosis. Studies are carried out both in vitro and in vivo. In vitro we are investigating the regulation of extracellular matrix components using lines of glomerular cells which we have isolated and characterized. In vivo, we use both transgenic and naturally occuring genetically-induced disease models. In addition, we have developed a new technique consisting of microdissection of single mouse glomeruli. We have also developed a competitive assay for the quantitation of the polymerase-chain reaction. This technique is now being applied to models of glomerulosclerosis recently developed in the laboratory including the glomerulo-sclerosis in mice transgenic for growth regulatory peptides and non-obese diabetic mice in which the onset of clinical diabetes mellitus has been synchronized. Current investigators are: L. Striker, G. Striker, T. Doi, E. Peten, S. Elliot, M. Carome, C. Pesce, K. Schmidt, and D. Yang.

Glomerulosclerosis

A. In Vivo Studies.

1. Mice transgenic for GH, GHRF and IGF-I: We found that mice transgenic for GH and GHRF develop fatal kidney disease. This is characterized by severe progressive glomerulosclerosis with an increase in the size of the glomeruli. This finding suggests that overexpression of GH may have effects in addition to those on growth. We found that there was an increase in the mRNAs coding for collagen type I, type IV and laminin B1 in the kidneys, utilizing an analysis of mRNA species in individual, isolated mouse glomeluli by reverse transcription and the polymerase chain reaction. The elucidation of the pathogenetic events in this model may lead to further understanding of the general pathogenesis of glomerulosclerosis, the most common of renal failure in humans (T. Doi, L. Striker, E. Peten, G. Striker).

2. Nonobese diabetic (NOD) mice: NOD mice develop autoimmune insulin-dependent diabetes mellitus (IDDM). We compared the glomerular size, morphology, composition of the glomerulosclerosis, and urinary abnormalities in mice with and without overt diabetes. Hyper-glycemia was rapidly followed by an increase in glomerular size, mesangial sclerosis, and proteinuria. We are synchronizing the onset of clinical diabetes mellitus with streptozotocin injections and assessing matrix synthesis in isolated cells, isolated glomeruli, and total kidney. These mice are the only good model of nephropathy in a genetically determined model of (IDDM). This study suggests that hyperglycemia triggers the rapid development of glomerular lesions, if the glomeruli have a genetic propensity to develop sclerosis. (T. Doi, D. Yang, L. Striker, G. Striker)

3. Kidney disease in diabetic Pima Indians. We have undertaken a retrospective study of the glomerular lesions in diabetic Pima Indians. We have developed morphometric methods to measure glomerular size to determine whether glomerulosclerosis is associated with hypertrophy. (L. Striker, P. Bennett, G. Striker, C. Pesce, K. Schmidt)

4. Ablation model. Subtotal nephrectomy in rats is a well-studied model of progressive glomerulosclerosis. We are analyzing the early cellular events that may lead to glomerular scarring and found that there is an increase in glomerular and arterial cell turnover which occurs weeks before the appearance of extracellular matrix deposition. (L. Striker, C. Pesce, G. Striker)

B. In Vitro Studies.

1. Murine and human glomerular cells: We have developed lines of mouse epithelial, mesangial, and endothelial cells from normal mice and from several strains of transgenic mice. We have investigated their response to growth peptides and advanced glycosylation endproducts. Mesangial cells produce, IGF-I, IGF binding proteins, and synthesize collagen types IV and I as well as metalloproteinases I and their inhibitor (TIMP I). These mRNA species appear to be regulated by cell density. In addition, if cells are exposed to advanced glycosylation end-products there is a rapid increase in collagen synthesis. (T. Doi, S. Elliot, M. Carome, E. Peten, L. Striker, G. Striker)

2. Endothelial cells and insulin: We identified a receptor for insulin on the surface of a clone of endothelial cells from normal mice and have begun characterization of its function. (S. Elliot, L. Striker, G. Striker)

3. Analysis of lines of mesangial cells from mice that develop glomerulosclerosis: We developed mesangial cell lines from mice transgenic for growth hormone and from NOD mice. We are examining their cell cycle and synthesis/degradation of extracellular matrix. We postulate that phenotypic changes in the resident cells are characteristic of glomeruli in animals prone to glomerulosclerosis. (L. Striker, E. Peten, M.Carome S. Elliot, G. Striker)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43002-26 MD

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Structure, Secretion and Mechanism of Action of Parathyroid Hormone

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G.D. Aurbach, M.D.	Chief, MDB, NIDDK
OTHERS:	S. Doi, M.D.	Visiting Associate, MDB, NIDDK
	M.L. Brandi, M.D., Ph.D.	Visiting Associate, MDB, NIDDK
	K. Sakaguchi, M.D., Ph.D.	Visiting Associate, MDB, NIDDK
	Y. Fujii, M.D.	Visiting Fellow, MDB, NIDDK

COOPERATING UNITS *(if any)*

Endocrine Unit, Massachusetts General Hospital
Department of Medicine Yale University
National Institute of Dental Research, BRB

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Endocrine Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.6	3.6	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is the purpose of this project to study the secretion, function, and mechanism of action of parathyroid hormone, its relationship to human disease, and to develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine, rat and human parathyroid hormone have been determined. Synthetic polypeptides representing bovine rat and human parathyroid hormone have been synthesized. These molecules show all the biological properties of the native hormonal polypeptides. Highly sensitive radioimmunoassays for the hormone have been developed and are being modified further for improved clinical diagnostic parameters. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. Isolated parathyroid cells and culture systems have been developed that allow studies on secretory control of parathyroid hormone, and provide test systems to elucidate the pathophysiology of certain hypoparathyroid and hyperparathyroid states.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43003-26 MD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Mode of Action of Thyrocalcitonin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	J. Barsony, M.D.	Visiting Associate	MDB, NIDDK

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Mineral Metabolism Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It will also be of interest to solubilize the receptors and characterize them chemically. Calcitonin increases cAMP in MCF 7 breast cancer cells. At 300-fold lower concentration calcitonin decreases cAMP in these cells. The decrease in cAMP is prevented by preexposure of cells to agents that interfere with inhibitory guanyl regulatory proteins. Intracellular compartmentalization of cAMP accumulation after calcitonin has been imaged after microwave fixation of cells. The cAMP accumulates initially along the plasma membrane but within 1 to 3 minutes accumulates much closer to the nucleus.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43006-16 MD

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of Hyperparathyroidism: Etiology, Diagnosis and Treatment

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G.D. Aurbach, M.D.	Chief, MDB, NIDDK
OTHERS:	S.J. Marx, M.D.	Chief, Min. Metab. Sec., MDB, NIDDK
	M.L. Brandi, M.D., Ph.D.	Visiting Associate, MDB, NIDDK
	K. Sakaguchi, M.D., Ph.D.	Visiting Associate, MDB, NIDDK
	A. Spiegel, M.D.	Chief, MPB, NIDDK

COOPERATING UNITS *(if any)*
Radiology Department, CC; Surgery Branch, NCI; Digestive Diseases Branch, NIDDK

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Endocrine Regulation Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent or recurrent hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house clinical studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, ultrasonography, radiothallium scanning, magnetic resonance imaging, CAT scanning, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction. In vitro evaluation of parathyroid and other endocrine tissue involves tissue culture, chemistry and determination of linkage with DNA or RNA probes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43008-10 MD

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Vitamin D Resistance and Related Disorders

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	J. Barsony, M.D.	Visiting Associate	MDB, NIDDK
	W. McKoy	Chemist	MDB, NIDDK
	C. Smith, Ph.D,	IRTA	MDB, NIDDK

COOPERATING UNITS *(if any)*

Metabolism Unit, Beilinson Hospital, Petah Tiva, Israel	(U. Liberman)
Cell Biology Department, Baylor University	(J.W. Pike)
Biochemistry Department, University of Wisconsin, Madison	(H.F. DeLuca)
Hormone Action and Oncogenesis Sectionk NCI	(G. Hager)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.2	0.8

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

With recognition that vitamin D is the precursor for 1,25-dihydroxyvitamin D, it has become possible to characterize defects in the activation (1-hydroxylation) of vitamin D and defects in the target action of activated (1,25-dihydroxy)vitamin D. We have demonstrated a broad spectrum of manifestations of hereditary resistance to 1,25(OH)2D ranging from infantile rickets with alopecia and no intestinal response to calciferols to adult onset osteomalacia with satisfactory intestinal response to high doses of calciferols and with no epidermal abnormalities. This syndrome usually results from a mutation in the gene for the vitamin D receptor. Skin fibroblasts from all subjects with hereditary resistance to 1,25(OH)2D display abnormalities in this effector system, and defects in many discrete steps of this pathway have been identified with these cells. Cells with mutations in the 1,25(OH)2D effector pathway can be used to explore mechanisms of calciferol action. They have been used to establish that the 1,25(OH)2D receptor mediates an extremely rapid (1-3 minutes) rise of cyclic GMP in response to 1,25(OH)2D3 and that certain receptor mutations compromise many receptor functions but allow another function to be retained normally. This establishes that 1,25(OH)2D receptors couple to different responses by distinct mechanisms.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43009-06 MD

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Mineral Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	S.J. Bale, Ph.D.	Senior Staff Fellow	EEB, NCI
	W. McKoy	Chemist	MDB, NIDDK
	G. Aurbach, M.D.	Chief	MDB, NIDDK
	E. Friedman, M.D.	Visiting Fellow	MPB, NIDDK
	A. Spiegel, M.D.	Chief	MPB, NIDDK
	J. Norton, M.D.	Head, Metabolism Sec.	SB, NCI
	L. DeMarco, M.D., Ph.D.	IRTA	MDB, NIDDK

COOPERATING UNITS *(if any)*

EEB, SB, NCI, MPB

Belvedere Medical Center - Carlisle, PA (J. Green)

Genetics Department - Yale University (A. Bale)

Department of Endocrinology - University of Florence Italy (ML Brandi)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Disorders of mineral metabolism have been evaluated with methods extending from epidemiology to cellular and molecular biology. Two forms of familial hyperparathyroidism have been characterized in detail. Familial hypocalciuric hypercalcemia is an autosomal dominant trait associated with abnormal interactions with calcium in parathyroid and kidney. Familial multiple endocrine neoplasia type 1 (FMEN1) is an autosomal dominant trait causing hyperfunction of parathyroids, pancreatic islet and anterior pituitary. It is associated with gradual but abnormal proliferation of the tissues affected. Genetic linkage studies in a large kindred have localized the MEN1 gene to the long arm of chromosome 11. Plasma from affected persons shows high mitogenic activity upon cultured bovine parathyroid cells. This mitogenic activity in plasma may contribute to primary hyperparathyroidism in FMEN1. Analysis of blood and parathyroid tumor DNA has revealed that FMEN1 parathyroids often show clonal loss of alleles in the region of the FMEN1 gene on chromosome 11. Thus the FMEN1 gene functions as a tumor suppressor gene, analogous to the retinoblastoma gene. Analysis of sporadic parathyroid adenomas revealed that 25% showed allelic loss in a similar region. Thus the clonal inactivation of the FMEN1 gene may also be a contributing factor in many sporadic parathyroid adenomas. Tumors with allelic loss along chromosome 11 are being used to define a minimum region of loss in efforts to identify the FMEN1 gene locus.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43200-12 MDB

PERIOD COVERED
Obtober 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Disorders of Immune Regulation in Patients with Systemic Lupus Erythematosus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	G. C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	A. Patel	Biologist	MDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Kidney Disease Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.8	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with systemic lupus erythematosus have been found to have various disturbances of the cell-mediated immune response. Cellular aberrations include enhanced spontaneous B lymphocyte activity with abnormal triggering in vitro, deficient immunoregulatory T lymphocyte circuits, deficient cytotoxic responses, including natural killer cell activity, alloantigen and viral cytotoxicity, and abnormal production of and response to different lymphokines as well as increased expression of proto-oncogenes in highly activated peripheral blood lymphocytes.

CD4+ and CD4/CD8+ T lymphocyte receptor cells and cell lines from patients with active lupus nephritis provide help to autologous B lymphocytes to produce nephritogenic antibodies. The goal of these studies is to further elucidate the mechanisms of these alterations of the immune system which are apparently involved in the pathogenesis of this disease.

The modulation of the above disturbances by immunosuppressive agents, i.e. corticosteroids and cyclophosphamide, is actively studied, aiming at the restoration of normal immune status in these patients.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-[illegible]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43201-07 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Production and Characterization of Nephritic Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	G. C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	R. Schwertz	Guest Researcher	MDB, NIDDK
	A. Patel	Biologist	MDB, NIDDK

COOPERATING UNITS *(if any)*

SUNY Medical Center, Syracuse, NY (Dr. R. Spitzer)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The nephritic factor (NeF) of the alternative pathway of complement has been found in the sera of patients with membranoproliferative glomerulonephritis (MPGN) and partial lipodystrophy and has been described as a factor which is able to induce cleavage of the third component of complement (C3) in normal human serum through the alternative pathway. It has been demonstrated that NeF binds to and stabilizes C3bBb (alternative C3 convertase). NeF appears to be antigenically and structurally similar to IgG and therefore it might be an autoantibody directed against C3bBb complex. The relation between the development of renal lesions and the NeF mediated persistent hypocomplementemia remains unexplained. B lymphocytes from patients with MPGN were used to establish cell lines secreting NeF of either IgG or IgM classes. Sera of patients with MPGN were found contain anti-idiotypic antibodies to NeF. We isolated 3 different anti-idiotypic antibodies and found that monoclonal and several polyclonal NeF share at least one idiotope. To verify this observation we are in the process of repeating these experiments using hetero-anti-idiotypic antibodies. Nucleotide sequencing of different NeF will answer the question whether NeF are direct products of germline genes or have undergone mutations as a result of antigenic stimulation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43202-08 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Regulation of Human Immune Response by Complement

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	G. C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Complement factors and breakdown products acting through cell surface membrane receptors block the differentiation of human B lymphocytes into immunoglobulin secreting cells. Complement receptors are associated with B cell surface immunoglobulin under certain circumstances. Furthermore, complement receptor expression is cell-cycle dependent.

Monovalent ligands inhibit while polyvalent enhance the anti-IgM induced human B cell increase in intracytoplasmic calcium ion concentration and cell proliferation.

Understanding of the mechanism of regulation of immune responses by complement and the role of complement receptors on human B cells is crucial for the understanding of the immunopathogenesis of autoimmune diseases since they are frequently associated with complement activation, depression of complement factor levels and changes in complement receptors.

U.S. GOVERNMENT PRINTING OFFICE 1991 O - 4[illegible]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK43204-11 MDB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunosuppressive drug therapy in lupus glomerulonephritis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. E. Balow	Senior Investigator	MDB, NIDDK
Others:	H. A. Austin	Medical Officer	MDB, NIDDK

COOPERATING UNITS *(if any)*

NIAMS (J. Klippel, P. Plotz, A. Steinberg, R. Wilder).

LAB/BRANCH
Metabolic Disease Branch

SECTION
Kidney Disease Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The optimal treatment of the proliferative forms of kidney disease associated with systemic lupus erythematosus is controversial. The efficacy of intensive, intermittent immunosuppressive drug therapy is being evaluated in patients with active lupus glomerulonephritis. A comparison is being made between intermittent pulse doses of corticosteroid and cyclophosphamide, as well as between a short and long course of pulse cyclophosphamide. Patients with renal biopsy documented active glomerulonephritis were treated with prednisone and randomized to receive concomitantly (a) intravenous pulse methylprednisolone monthly for 6 months, or (b) intravenous pulse cyclophosphamide monthly for 6 months, or (c) pulse cyclophosphamide monthly for 6 months followed by a maintenance regimen of pulse cyclophosphamide every 3 months for an additional two years. Results to date indicate a statistically significant, higher probability of progressive renal insufficiency in patients treated with pulse methylprednisolone than in patients treated with pulse cyclophosphamide.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43205-14 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Renal biopsy pathology in systemic lupus erythematosus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Balow	Senior Investigator	MDB, NIDDK
Others:	H. Austin	Medical Officer	MDB, NIDDK

COOPERATING UNITS *(if any)*

Armed Forces Institute of Pathology, Washington, DC
(T. Antonovych, S. Sabnis).

LAB/BRANCH

Metabolic Disease Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Diverse pathogenetic factors are operant in systemic lupus erythematosus and lead to different forms of lupus nephritis. Detailed analysis of renal biopsy pathology is being conducted on specimens from patients with systemic lupus erythematosus.

Biopsies are classified by standard major category of lupus nephritis, as well as scored on a semiquantitative scale for specific histologic changes which indicates the extent and severity of active inflammatory lesions and of chronic atrophic, fibrosing and sclerosing features. The patterns of immune complex deposition and lymphoid cell interaction with different segments of the nephron are being investigated by immunohistologic techniques and electron microscopy. Computer-based morphometric techniques are being developed to analyze more precisely the changes in renal pathology during the course of lupus nephritis.

These approaches have facilitated the analysis of the effects of various types of immunosuppressive agents used to halt the progression of lupus nephritis and they will enhance our understanding of the pathogenesis of the kidney disease of systemic lupus erythematosus.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43210-06 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Glomerular Disease in Transgenic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Chief	RCBS, MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	K. McKay	Medical Staff Fellow	MDB, NIDDK

COOPERATING UNITS *(if any)*

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-DK 43211-07 MD

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Morphometry of Renal Lesions in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-PI:	G. Striker	Director	DKUHD, NIDDK
	C. Pesce	Visiting Associate	RCBS, MDB, NIDDK
	K. Schmidt	Medical Staff Fellow	DCBD, NCI

COOPERATING UNITS (if any)

Epidemiology and Clinical Research Branch, NIDDK, Phoenix Arizona
(Peter Bennett)

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Renal Cell Biology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.90	0.90	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

Controversy exists as to whether the nephropathy in NIDDM is similar to that in IDDM, since the onset of NIDDM is difficult to ascertain. The NIDDK epidemiologic survey of Pima Indians provides a unique opportunity for the early detection of NIDDM and its associated nephropathy, a long-term follow up of both aspects of the disease, the opportunity to study their natural histories. However, there has been question as to whether the renal disease in the Pima resembles that in caucasians. Therefore, we studied autopsy kidney specimens from diabetic and control Pimas by morphometric techniques. The weighted mean of the area and a size-class distribution of the glomeruli were calculated with a computer-assisted planimeter in a series of autopsy kidney specimens of diabetic and non-diabetic Pima Indians. These morphometric variables were also related to the severity of histologic glomerular lesions, graded on a four class scale. Glomerular size did not differ in the two groups, nor were differences in the size distribution discernible among the classes of histologic lesions. A similar pattern was found in the Causcasian population affected by type-II DM. These data are in contrast to type I DM where there is an early increase in glomerular size followed by a late decrease in glomerular size as end-stage renal disease supervenes. Thus, the renal lesions in diabetic Pima indians resemble those in the caucasian population.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43214-07 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Studies of Glomerular Cells Derived from Transgenic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-PI:	S. Elliot	Biologist	RCBS, MDB, NIDDK
	E. Peten	Special Volunteer	RCBS, MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.22	0.22	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Glomerular scarring (glomerulosclerosis), a slowly progressive disease, is the most common cause of kidney failure. Current models of glomerulosclerosis (GS) have yielded little information about the cellular and molecular abnormalities that are critical in the initiation and progression of this disease since they most often are models of acute inflammation. For this reason we constructed models of slowly progressive disease of a non-inflammatory type. The complexity of the kidney and glomerulus makes isolation and examination of pure cultured populations of glomerular cells an attractive method for beginning to address the pathogenesis of GS. Mice transgenic for the early region of simian virus 40 (SV40) and for growth hormone develop slowly progressive GS. As there are no evident extrarenal sources of injury, and since expression of the foreign DNA has been documented to occur in whole kidney, we postulated that the glomerular disease may be secondary to expression of the foreign DNA by glomerular cells in vivo. We have isolated lines of glomerular endothelial, mesangial, and epithelial cells from transgenic mice and have isolated pure cultures of mesangial, endothelial cells and epithelial cells from their normal litter-mates. Our data from this in vivo model indicates that proliferation of glomerular cells is an early event in the development of GS in transgenic mice.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 43217-06 MD

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Renal Lesions in Leukemias, Lymphomas and Carcinomas

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L Striker	Chief	RCBS, MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK

COOPERATING UNITS *(if any)*
National Cancer Institute, Bethesda, Maryland (M. Merino, W. Travis).

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Renal Cell Biology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD. 20892

TOTAL MAN-YEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

PHS 6040 (Rev. 1/84) GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43221-06 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biology of Insulin Receptors in Glomerular Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Elliot	Biologist	RCBS, MDB, NIDDK
Others:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md.

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
0.12	0.12	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our studies demonstrated specific insulin receptors on isolated glomeruli. We showed that the majority of the glomerular surface receptors on endothelial cells. The endothelium acts as an interface between the blood and the glomerular mesangium, therefore insulin binding to endothelial cell receptors may affect the mesangial cells. Insulin may mediate the production of nitric oxide, a potent vasoconstrictor for mesangial cells, through stimulation of amino acid uptake. To understand if the insulin receptor is dysfunctional in diabetes, we are currently isolating endothelial cells from non-obese diabetic mice for further studies on the insulin receptor.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43222-06 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Pathogenesis of Murine Lupus Nephritis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P. I.:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	D. T. Boumpas	Visiting Scientist	MDB, NIDDK
	A. D. Patel	Biologist	MDB, NIDDK

COOPERATING UNITS *(if any)*

Armed Forces Institute of Pathology; Washington, DC (Drs. T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.35	0.35	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Investigations of the pathogenesis and treatment of lupus nephritis are facilitated by the availability of inbred strains of mice that develop disease similar to human systemic lupus erythematosus. The natural evolution of the diverse histologic features of murine lupus nephritis has been studied to delineate the types of glomerular and tubulointerstitial lesions. Innovative treatment strategies will be studied to refine our approach to this disease. The impact of biologic response modifiers on immunologic features will be investigated as well. Clinical, histologic and immunologic outcome parameters will be evaluated including detailed studies of renal morphology, and the characteristics of spleen lymphocytes employing flow cytometry, measures of immunoglobulin gene expression, and in vitro assays of alterations in humoral and cell mediated immune regulation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43224-05 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Membranous Lupus Nephropathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	K. MacKay	Expert	MDB, NIDDK

COOPERATING UNITS *(if any)*

CC (E. Vaughan); NIAMS (J. Klippel); Stanford University; Stanford, CA (B. Myers). Armed Forces Institute of Pathology, Washington, D.C. (Drs T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Disease Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.45	0.45	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is currently unknown whether therapeutic intervention will alter the course of membranous lupus nephropathy. In the present study, the efficacy and toxicity of three immunosuppressive drug regimens administered over a 12 month period will be evaluated in patients with membranous lupus nephropathy. Detailed tests of renal function (including radiolabelled compounds for glomerular filtration and renal plasma flow rates), glomerular permselectivity (using fractional clearance of graded dextrans) and kidney biopsy morphology will be performed at the beginning and end of treatment. Patients with systemic lupus erythematosus, nephrotic range proteinuria and biopsy documented membranous nephropathy will be randomized to receive: a) alternate day prednisone alone (control group), b) alternate day prednisone plus intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) alternate day prednisone plus oral cyclosporin A up to 200 mg per square meter body surface area daily. Lupus disease activity, renal function tests and drug toxicities will be monitored closely. Analysis will include comparison of the numbers of favorable outcomes of glomerular filtration rate, renal plasma flow, permselectivity, glomerular pathology and drug-related toxicities appearing in each treatment group.

U.S. GOVERNMENT PRINTING OFFICE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43225-04 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Glomerular Lesions in Mice Transgenic for GH

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Others:	T. Doi	Visiting Scientist	RCBS, MDB, NIDDK
	E. Peten	Special Volunteer	RCBS, MDB, NIDDK
	D. Yang	Guest Researcher	RCBS, MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK

COOPERATING UNITS *(if any)*

John Kopchick - Ohio University, Dept. of Zoology, Athens, Ohio 45701

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.10	1.10	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Increased glomerular size occurs in the presence of normal maturation, following unilateral nephrectomy in humans and animals, and in disease states such as diabetes mellitus. The hormonal regulation of compensatory hypertrophy is not fully understood, however total kidney IGF-I mRNA levels are increased following unilateral nephrectomy. This suggests a role for this hormone in hypertrophy of the adult kidney as well as in normal development. There are abnormalities in the circulating levels of GH in some diseases associated with increases in glomerular extracellular matrix and cell number, such as diabetes mellitus. The availability of transgenic mouse strains expressing elevated levels of IGF-I, GH, and GHRF provides an opportunity to study the renal effects of chronic hormone exposure. We have observed that mice containing an MT-I IGF-I fusion gene develop large glomeruli which are normal in appearance, whereas those transgenic for either growth hormone or growth hormone releasing factor have large glomeruli which are hypercellular. In addition, progressive glomerulosclerosis develops in the GH and GHRF transgenic mice. We further showed that there was an upregulation of mRNA for extracellular matrix components in the kidneys of the GH animals, which persisted late in the course of the disease. Surprisingly, proliferation also persists at late stages, when glomerular scarring was essentially endstage.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43227-04 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of IGF-1 in the Biology of Mouse Glomerular Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. Elliot	Biologist	RCBS, MDB, NIDDK
Co-Inv.:	G. Striker	Director	DKUHD, NIDDK
	L. Striker	Senior Inv.	RCBS, MDB, NIDDK
	E. Peten	Special Volunteer	RCBS, MDB, NIDDK

COOPERATING UNITS (if any)

Derek LeRoith - Diabetes Branch, NIDDK

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md.

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.70	0.70	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The IGF-I axis has been implicated in the development of renal complications of diabetes. The glomerulosclerosis of diabetes is characterized by cell proliferation and accumulation of extracellular matrix in the mesangium. This suggests that there may be an intrinsic defect of mesangial cell behavior. The non-obese diabetic mice (NOD) develop glomerular lesions early after the onset of diabetes. To study the role of the IGF-I axis in a model of spontaneous diabetes, we developed new lines of mesangial cells derived from these animals. We examined the IGF-I receptor, IGF-I production, and IGF-I binding proteins of NOD mouse cells and compared them to controls. We previously demonstrated the presence of IGF-I receptors and the synthesis of IGF-I in glomerular mesangial cells. Preliminary studies suggest that NOD mesangial cells differ substantially from normal cells with respect to the IGF axis.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43228-04 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biology of Human Glomerular Mesangial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-PI:	S. Elliot	Biologist	RCBS, MDB, NIDDK
	E. Peten	Special Volunteer	RCBS, MDB, NIDDK
	M. Carome	Guest Researcher	RCBS, MDB, NIDDK
	T. Doi	Visiting Scientist	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.45	0.45	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mesangial cell hyperplasia is a feature of several human glomerular diseases. The cause of this increased cell number is unknown. We assessed human mesangial cells in vitro and found that they possessed an insulin-like growth factor-I (IGF-I) receptor consisting of α and ß units (Mr 130k and 90k respectively). Fifty percent inhibition of IGF-I specific binding to the receptor required 1×10^{-9}M IGF-I, 1×10^{-6}M insulin, and 1×10^{-7}M multiplication stimulating activity (MSA). Analysis of binding by the method of Scatchard revealed one type of IGF-I receptor with a $kd=1.35\times10^{-9}$M, and a number per cell of 1.04×10^{5}. Binding studies on whole glomeruli was of similar specificity and there were 7.17×10^{7} receptors per glomerulus ($kd=1.12\times10^{-9}$M). Examination of the effect of IGF-I on the cell cycle revealed that cells treated with platelet derived growth factor (PDGF) had a rapid ^{3}H-thymidine response which was abolished by anti-PDGF antibody. Finally, PDGF increased RNA and protein sysnthesis, which was not enhanced by IGF-I. In summary, human mesangial cells, and whole glomeruli, possess IGF-I specific receptors and IGF-I was found to act as a progression factor in the cell cycle.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43230-02 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Proteoglycan Production by Mouse Glomerular Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Striker	Director	DKUHD, NIDDK
Others:	K. McKay	Medical Staff Fellow	RCBS, MDB, NIDDK
	L. Striker	Chief	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*

Department of Cell Biology, Yale School of Medicine, New Haven, Connecticut (J. Stow, M. Farquhar).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, nIH, Bethesda, MD. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43231-03 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Idiopathic Membranous Nephropathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I. :	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	K. MacKay	Expert	MDB, NIDDK

COOPERATING UNITS *(if any)*

Stanford University, Stanford, CA (Dr. B. Myers); CC (E. Vaughan, Nursing); Armed Forces Institute of Pathology, Washington, DC (Drs. T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER
0.45	0.45	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Patients with idiopathic membranous nephropathy are being studied to evaluate the efficacy and toxicities of the addition of intermittent cyclophosphamide or daily oral cyclosporin A to alternate day oral corticosteroid therapy. Efficacy will be judged by determinations of effective renal plasma flow, glomerular filtration rate and glomerular capillary wall permselectivity performed with dextran and urine protein (albumin and immunoglobulin) clearance techniques. Kidney biopsy morphology (including morphometric analysis) will be examined at the beginning and at the end of treatment as part of detailed studies of structure-function relationships and the efficacy of various therapeutic modalities.

Patients with membranous nephropathy and 2 or more grams per day of proteinuria will be treated with alternate day prednisone and will be randomized to receive: a) no additional therapy (control group), b) intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) oral cyclosporin A up to 200 mg per square meter body surface area daily for a total of 11 months. Analysis will include comparison of the number of favorable outcomes of glomerular function and pathology as well as drug-related toxicities observed in each treatment group at the end of the 12 months of study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43232-02 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Renal Lesions in the Ablation Model: Role of Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-PI:	G. Striker	Director	DKUHD, NIDDK
Collab:	C. Pesce	Visiting Associate	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*

Washington University, St. Louis, Missouri (S. Klahr, M. Purkenson)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md.

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
0.55	0.55	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Subtotal nephrectomy in rats, the so-called ablation model, leads to progressive destruction of the kidney and the development of chronic renal failure. We postulated that the progressive glomerular lesions were due to an early increase in the turnover of glomerular resident cells. This abnormal glomerular growth results in an increase in the glomerular volume which is detectable using morphometric measurements. We have performed autoradiographic studies, using ^{3}H-thymidine, and found that within two days following subtotal nephrectomy there was an increase in the glomerular mitotic index, as well as an increase in the turnover of the cells forming the arterial wall. These findings suggest that a dysregulation of cell growth is an early event in the development of glomerulosclerosis following reduction in renal mass.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 930

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43233-02 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Identification of IGF-1 Binding Proteins by Mesangial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Striker	Director	DKUHD, NIDDK
Co-PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
	S. Elliot	Biologist	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*

David Clemmons, Dept. of Medicine, University of North Carolina

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md.

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
0.26	0.26	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We investigated the synthesis of IGF-I binding proteins in vitro by normal mouse mesangial cells. The supernatant from mesangial cells contained a single species of binding protein, with a molecular weight of 30 kD. This protein, known as BP2, was not glycosylated. It was found to be released in higher amounts in cells at lower density than in confluent cells. It was also found that the cells expressed mRNA for this binding protein. The binding protein release was coordinately regulated with the production of IGF-I by the cells. Thus, IGFBP2 could participate in the control of mesangial proliferation. In addition, diseases associated with an increase in glomerular cell turnover could be partly due to a dysregulation in IGFBP production and release.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43234-02 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interactions between TGF-B and Glomeruli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	K. MacKay	Expert	MDB, NIDDK
Others:	April R. Robbins		LBM, NIDDK
	David Danielpour		LC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.80	0.80	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Increases in glomerular cellularity and accumulation of extracellular matrix material are prominent histologic findings in a number of clinical and experimental glomerular diseases. Transforming growth factor-ß (TGF-ß) has been identified as a potentially important modulator of glomerular pathology based on its demonstrated ability to regulate proliferation and extracellular matrix synthesis by cultured glomerular cells. In addition, we previously found that normal rat glomeruli contain high concentrations of TGF-ß1 and TGF-ß2 and that glomeruli possess unique TGF-ß binding proteins or receptors. The goal of these studies is to better understand the actions and mechanisms of action of TGF-ß in the glomerulus. The current focus of these studies is on the TGF-ß binding proteins and/or receptors which are present in glomeruli.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43235-02 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Glomerular Lesions in Non-obese Diabetic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-Inv.:	G. Striker	Director	DKUHD, NIDDK
	T. Doi	Visiting Scientist	RCBS, MDB, NIDDK
	D. Yang	Guest Researcher	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*

M. Hattori - Joslin Diabetic Center, Boston, Massachusetts

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

NOD mice spontaneously develop insulin-dependent diabetes mellitus (IDDM) secondary to immunologically-mediated beta cell destruction in pancreatic islets. Shortly after the appearance of diabetes, NOD mice developed renal lesions consisting of diffuse mesangial sclerosis, thickening of glomerular basement membranes, and the development of albuminuria. These lesions closely mimic those of IDDM in humans. Morphometric analysis showed that the kidney weight and glomerular size were increased in diabetic mice compared to non-diabetic mice, and further, that the ratio glomerular volume/kidney weight was also elevated in diabetic mice. These findings suggest that this disproportionate increase in glomerular size may play an important role in the development of diabetic nephropathy. Preliminary analyses of mRNA levels of matrix proteins, assessed by the RNase protection assay, reveal relative increases in α1 type IV collagen, laminin B1, and heparan sulphate proteoglycan. Finally, since the onset of diabetes is variable in NOD mice, we have begun experiments in which we have synchronized the induction of diabetes by the injection of streptozotocin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-43236-01 MD

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Advanced Glycosylation End Products, Effect on Mesangial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-PI:	G. Striker	Director	DKUHD, NIDDK
	T. Doi	Visiting Scientist	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*

Laboratory of Medical Biochemistry, Rockfeller University: Dr. H. Vlassara

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md.

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.60	0.60	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

End-stage glomerulosclerosis constitutes a major complication of diabetes mellitus. The fact that the glomerular lesions of both type I and type II diabetes are similar suggests that abnormalities in glucose metabolism may participate in their development. Hyperglycemia leads to the accumulation of advanced glycosylation end-products which participate in abnormal, non-metabolizable cross-linking of extra-cellular matrix components. Their accumulation may contribute to the sclerosis observed in diabetics, since AGEs trigger a large number of biological reactions through surface receptors that have been characterized on macrophages, endothelial cells, and human and rat mesangial cells. Mesangial cells plated on various components of glycosylated extracellular matrix produce an excess of fibronectin. Using normal mouse mesangial cells, we investigated the effect of AGE on the synthesis of the basement membrane components. Cells plated on AGE showed increased mRNA levels of collagen type IV, proteoglycan heparan sulfate, and laminin A and B chains using the RNase protection assay. There was also an increased release of collagen type IV in the medium. The rate of transcription, measured by nuclear run-off assays, was also stimulated in cells plated on glycosylated bovine serum albumin. These observations provide further evidence that the accumulation of extra-cellular matrix components in diabetics is regulated at the gene level.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43[illegible]37-01 MD

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (*80 characters or less Title must fit on one line between the borders.*)
Production of Metalloproteinases and TIMP1 by Glomerular Cells

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Carome	Guest Researcher	RCBS, MDB, NIDDK
Co-Inv.:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	E. Peten	Special Volunteer	RCBS, MDB, NIDDK

COOPERATING UNITS (*if any*)
Department of Pathology, NCI: Dr. William Steten-Stevenson

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Renal Cell Biology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Betehsda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.80	0.80	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Glomerulosclerosis occurs in a large number of human kidney diseases including diabetic nephropathy. It consists of the accumulation of extracellular matrix (ECM) within the glomerulus. Sclerosis may be mediated by dysregulation of both synthesis and degradation of ECM. A large family of matrix metalloproteinases as well as tissue inhibitors of metalloproteinase (TIMPs) play a role in the degradative process. Preliminary work reveals that: 1) normal mouse mesangial cells in culture secrete a 72 kD and a 92 kD gelatinase (type IV collagenase) as well as TIMP1; 2) mesangial cells derived form NOD mice and mice transgenic for bovine growth hormone (bGH) secrete only the 72 kD gelatinase; and 3) TIMP1 mRNA levels are modulated by cell density.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43238-01 MD

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Expression in Microdissected Mouse Glomeruli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. Striker	Senior Investigator	RCBS, MDB, NIDDK
Co-Inv.:	G. Striker	Director	DKUHD, NIDDK
	E. Peten	Special Volunteer	RCBS, MDB, NIDDK

COOPERATING UNITS *(if any)*
Arlyn Garcia Perez: Laboratory of Kidney and Electrolyte Metabolism - NHLBI
Brian Martin: Clinical Neurosciences, NIMH

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Renal Cell Biology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.70	0.70	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to determine the phenotype of extracellular matrix molecules in normal adult mouse glomeruli. We used the increased sensitivity afforded by the polymerase-chain reaction to assess type I and type IV collagen mRNA in freshly microdissected normal adult mouse glomeruli. RT-PCR reactions for mRNA encoding these components were also performed using mesangial cell lines previously isolated from the same strain of mice. Type IV collagen mRNA was easily detectable in normal adult mouse glomeruli as well as in the cell lines. On the other hand, type I collagen mRNA was not detected in the glomeruli, despite increasing the number of PCR cycles from 25-45 (roughly a 1000 fold increase in sensitivity). Utilizing the same primers, type I collagen mRNA was easily demonstrable in two lines of mouse mesangial cells. These experiments support data in both humans and experimental models which failed to demonstrate type I collagen by immunofluorescence microscopy in normal glomeruli whereas type IV collagen was present in large amounts. The current study provides evidence that the expression of types I and IV collagen in normal glomeruli is regulated at the pretranslational level in vivo.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43239-01 MDB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of glucocorticoids on T cell activation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	D.T. Boumpas	Visiting Scientist	MDB, NIDDK
Others:	J.E. Balow	Senior Investigator	MDB, NIDDK
	F. Paliogianni	Visiting Fellow	MDB, NIDDK
	S. Ahuja	Visiting Associate	MDB, NIDDK

COOPERATING UNITS *(if any)*
Naval Medical Research Institute; Bethesda, MD (H. Yamada)
Arthritis and Rheumatism Branch (NIAMS); Bethesda, MD (G. Poy)

LAB/BRANCH
Metabolic Disease Branch

SECTION
Kidney Disease Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.35	1.35	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Activation of T cells is a complex process involving cell membrane, cytoplasmic and nuclear events. These events enable T cells to proliferate and exert their immunoregulatory function. Glucocorticoids inhibit T cell activation and thereby interfere with numerous immunoregulatory functions carried out by activated T cells. The goal of these studies is to better understand the actions and mechanisms of action of glucocorticoids on this process. We will study their effects on early (tyrosine kinase and protein kinase C activation, and calcium influx), intermediate (production of transcription factors) and late activation events (lymphokine gene transcription). Furthermore, their post-transcriptional effects on the expression of two important lymphokines for the pathogenesis of immune mediated renal disease, namely interleukin-2, transforming growth factor-beta and their receptors, will also be examined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43240-01 MDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transcriptional regulation of immunoglobulin genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	D.T. Boumpas	Visiting Scientist	MDB, NIDDK
Others:	F. Paliogianni	Visiting Fellow	MDB, NIDDK

COOPERATING UNITS *(if any)*

Arthritis and Rheumatism Branch, NIAMS; Bethesda, MD (G. Poy)
University of Patras, Patras, Greece (E. Anastassiou)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.50	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Reduction of pathogenic autoantibodies is the main objective in the therapy of immune mediated renal diseases. Although rates of immunoglobulin synthesis can be regulated at multiple levels, most attention has been focused on transcription, as this seems to be the limiting step in most situations that have been examined. The goal of these studies is to examine the ability of a variety of agents (known to regulate the activation, proliferation and differentiation of B cells) to modulate the nuclear transcription of immunoglobulin heavy and light chain genes. These agents include antibodies to surface immunoglobulin, cytokines (interleukins, interferon, transforming growth factor), bacterial mitogens, pharmacologic agents (ionomycin, phorbol esters, prostaglandins) and immunosuppressive drugs (glucocorticoids, cyclosporin, cyclophosphamide). The mechanism of action of these agents will be explored by examining their effects on the production of transcription factors necessary for immunoglobulin gene transcription. Particular emphasis will be placed in detecting similarities, differences, synergism or antagonism in their effects.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

Dr. Jacob Robbins and Dr. Jan Wolff served on a team representing The Department of Energy under the bilateral United States - USSR agreement on Atomic Energy. Its purpose is to initiate a scientifically sound epidemiologic study of the thyroid effects of radiation resulting from the nuclear power station accident in Chernobyl, Ukraine, May 1986. Workshop meetings have been held in Bethesda in May 1990, and in Chernigov and Kiev, Ukraine and Minsk, Byelorussia in December 1990, and again in Kiev and Minsk in June 1991 in order to develop a protocol acceptable to both the US and the USSR and to submit this proposal for funding by the DOE. This nuclear accident, which is the largest yet to occur, resulted in significant thyroid radiation exposure in about 70,000 inhabitants of Ukraine and Byelorussia, and offers an opportunity to define the risk for developing thyroid nodules and cancer from exposure to I-131. At present there is no accurately defined risk estimate for I-131 induced thyroid cancer.

Dr. Vera Nikodem was an invited speaker at the UCLA-Keystone Conference, March 1991, on Molecular Approaches to Thyroid Hormone Action. She delivered a key paper entitled "Thyroid hormone gene products and their trans-activating functions in cell culture".

Dr. Kevin Petty, a Medical Staff Fellow with Dr. Nikodem, received the Young Scientist award of The American Federation for Clinical Research for his work on transacting factors involved in gene regulation by thyroid hormone.

Visiting Fellows from Czechoslovakia, France, Greece, India, Italy, Japan and the USSR participated in the work of the Branch during this year.

I. Thyroid Biochemistry and Pathophysiology

A. Thyroid Hormone - Protein Interactions

It was previously shown that the gel filtration profile of the lipoproteins from normal human plasma equilibrated with ^{125}I-T4 separates several HDL particles which bind T4. Most of the T4 is recovered in the smaller size HDL subfraction, corresponding to HDL^3, and is mediated by T4 binding to the major apolipoprotein A-I. In further studies of multiple preparations of HDL$_2$, as well as the heavier sulfraction, HDL$_3$, it was found that HDL$_3$-apoA-I binds T4 more avidly and more consistently than HDL$_2$-apoA-I. Inhibition of T4 binding by T4 itself and other competitors was variable and was generally more effective with HDL$_2$. This suggests that the apoA-I in the two major HDL subclasses exists in different conformations having different abilities to bind T4.

Further studies showed that photoaffinity labeling of intact HDL with T4 resulted in the labeling of several other apolipoproteins including apo(a), apoB 100, apoA-II, apoA-IV, apoC and apoE. Thus, the ability to interact with T4 is a general property of apolipoproteins. Since apolipoproteins are involved, through specific receptors, in cellular entry and removal of lipids, it is apparent that T4 entry and removal may also be affected by its interaction with apolipoproteins (Benvenga, Robbins).

The major transport protein for T4 in human plasma is thyroxine-binding globulin (TBG) which is synthesized in hepatocytes. TBG is homologous to the "acute phase proteins", the serine antiproteases, the synthesis of which is increased by cytokines. Therefore, it was of interest to test the effect of the cytokine IL-6 on TBG synthesis. It was found that IL-6 increased the synthesis of antichymotrypsin and antitrypsin in HepG2 cells by a factor of 2, but caused a 30% decrease in the synthesis of TBG. A similar decrease was found in the homologous cortisol binding globulin (CBG) as well as in the other T4 transport proteins, transthyretin and albumin. By hybridization with DNA, it was shown that the decrease in TBG synthesis was at a pretranslational level. Thus, despite its homology with serine antiproteases, TBG is not an acute phase protein, but rather it is a negative acute phase reactant (Bartalena, Robbins).

B. Thyroid Hormone Action

The rat nuclear thyroid hormone receptor alpha (rTRα), produced in large quantity by a recombinant baculovirus in Sf9 insect cells, was used to study interactions of the receptor with thyroid hormone, and with other nuclear proteins. Using various electrophoretic and cross-linking techniques, it was demonstrated that rTRα forms homodimers in solution and binds T3. Both the liganded and unliganded homodimer bind to an 18bp region of the promoter region of the malic enzyme gene (ME-TRE) as well as to the growth hormone gene palindromic TRE. Neither the monomer or higher polymers of rTRα were present, strongly suggesting that rTRα bound to the TRE as a homodimer may be the transcriptionally active form.

Further studies were done with H-2 RIIBP, a member of the steroid/thyroid hormone receptor superfamily that does not bind thyroid hormone. H-2 RIIBP binds to the major histocompatability complex class I gene, and was cloned by Dr. K. Ozato (NICHD), with whose collaboration this work was carried out. Cross-linking analysis showed that H-2 RIIBP formed homodimers as well as heterodimer with rTRα in solution. The heterodimer was more stable than either homodimer, and the heterodimer was shown to bind to the ME-TRE with higher affinity that the rTRα homodimer, while the H-2 RIIBP homodimer did not bind to the ME-TRE. It was also shown that both half-sites of the ME-TRE are required for binding and that thyroid hormone increases the binding affinity. It was further shown that both rTRα homodimer and the heterodimer bind only one mole of thyroid hormone. In addition, it was found, using ME-TRE linked either to thymidine kinase or chloramphenicol acetyltransferase expression vectors, that the heterodimer plus thyroid hormone was a more potent expression activator than the rTRα homodimer - thyroid hormone complex. These results suggest that formation of heterodimers between rTRα and nuclear proteins such as H-2 RIIBP may be important factors in thyroid hormone action (Hallenbeck, Nikodem).

Studies of the malic enzyme promoter element 2 (MPE-2) continued. This element is comprised of a direct repeat of the sequence CTCGCCACCC, and is similar to motifs found in the human c-fos and TGF-β promoters in which it functions as a transcriptional element responsive to the retinoblastoma gene product. Thus proteins interacting with this region of the ME gene may be important in the regulation of many other genes. Two such proteins have been obtained and partially characterized. They also interact specifically with ME-TRE. Thus the same or similar nuclear proteins bind to two seperate transcriptional cis-regulatory elements, one of which also binds the T3 receptor, and they may play important roles in the ME transcriptional response to thyroid hormone (Petty, Raptis, Nikodem).

The possible physiological significance of the two forms of thyroid hormone receptor, TRα and TRβ, and the effect of a non-hormone binding variant, TRαv, have been investigated with promoters of ME and MBP genes. The different effects observed indicate that the α and β receptor do not have redundant functions despite their similarity. The variant was able to either repress or enhance T3 activated expression, depending on the combination of genes and receptors that were used. Chimeric receptors were constructed with interchanged α and β DNA and ligand binding domains. These gave further evidence for variable responses dependent on the various combinations of elements in the reaction (Desvergne, Petty, Nikodem).

Studies have continued on the myelin basic protein (MBP) gene as a model of thyroid hormone action on the developing brain. It has now been shown by nuclear run off assays, using antisense MBP mRNA for hybridization, that the effect of thyroid hormone is to increase the transcription rate of the MBP gene. In transient transfection assays, it has been demonstrated that the thyroid hormone response element (TRE) of the MBP gene is located within 256bp of the start site in the 5' flanking region between -186 and -163, and contains an AGGAGA sequence in common with malic enzyme and myelin heavy chain TREs.

Further studies with the transient transfection systems showed that cotransfection of rTRα resulted in a 50% decrease of expression. Addition of thyroid hormone increased expression 4-fold (or 2-fold above the control). These responses in the MBP gene are similar to those seen with the malic enzyme gene (Farsetti, Nikodem, Robbins).

C. Studies in Thyroid Diseases

Studies are continuing on the diagnosis of thyroid cancer and its treatment with iodine-131. Six additional patients with high risk thyroid cancer have been recruited into the CRAFT protocol, designed to evaluate the adjuvant effect of the radiation enhancer, adriamycin. A total of 16 patients have entered the study and 5 have been randomized to receive the combination therapy. After a total of 10 doses of adriamycin (10mg/m^2), there have been no untoward responses. Although several patients showed a good response to therapy, it is too early to evaluate its effectiveness in comparison to I-131 alone.

In the study using I-131 to treat patients in whom serum thyroglobulin is elevated but the I-131 whole body scan is negative, a total of 13 patients have been treated on 22 occasions. Five pateints have reverted to a status in which the serum TG is 5ng/ml or lower, indicating that such therapy may be effective in eradicating persistent thyroid cancer (Ain, Shiver, Garcia, Robbins).

II. Mechanism of Cell Secretion

Studies have continued on microtubules, which are key elements in the mechanism of cell secretion. Proteolytic domains, monomer/monomer contact surfaces in the heterodimer, and the colchicine binding domain have been evaluated. Trypsin and chymotrypsin cleavage (at Arg 339 and Tyr 281, respectively) define domains that are exposed to solvent, while linker domains are inaccessible and maintain the dimer structure which can still polymerize into microtubules. The kinetics of the limited proteolysis of tubulin by subtilisin, and the observation that β-tubulin cleavage increases as tubulin concentration decreases, has permitted calculation of a dimer dissociation constant of 1.5 x 10^{-6}M. Similar values have been obtained by analysis of the shift in fluorencence wave length of nile red as well the more rigorous sedimentation analysis, which utilized a novel computerized short-column method. The letter gave a dissociation constant of 2 x 10^{-7}M (sackett, Wolff).

Further study of the role of fibrous elements in steroid hormone secretion by adrenal cells has been pursued using acrylamide, which had been shown to react preferentially with intermediate filaments. A comparison of the effects of acrylamide and ACTH on steroidogenesis indicates that acrylamide is more effective in causing rounding of Y-1 cells in monolayer culture but does not cause increased CAMP levels, as is typical of ACTH. Both agents enhance steroidogenesis to about the same degree and act at a step prior to pregnenalone formation. Since acrylamide does not affect microtubule assembly and the effects of acrylamide and ACTH are additive, it was concluded that these two agents stimulate steriodogenesis through different mechanisms (Shiver, Sackett, Wolff).

III. Mechanisms of Embryogenesis

Classical studies in amphibian embryology have demonstrated that an important step in anteroposterior axis formation is the polarization of the prospective mesoderm, followed by a transfer of this polarity to the overlying ectoderm during gastrulation. A number of specific genes, such as the homeobox gene, Xhox 3, are thought to play a role. In investigating this mechanism, a novel gene, Xenopus-posterior (Xpo) has been isolated and characterized. Xpo is activated at mid-blastula, is expressed maximally during mid-gastrulation to neurulation, and is restricted to the posterior dorsal mesoderm and ectoderm. A single copy of a finger motif of the CCHC type is located in the C-terminus and resembles motifs found in the essential nucleocapsid protein of retroviruses and in cellular nucleic acid binding protein (CNBP) that binds to the sterol regulatory element. Xpo expression is induced in ectodermal explants by basic FGF and polypeptide growth factors found in the medium of the Xenopus XTC cell line. It appears that Xpo may play an important role in the organization of the anteroposterior axis during development (Sato, Sargent-NICHD).

A PCR strategy has allowed the molecular cloning of a family of POU-domain genes, most of which are expressed during early embryogenesis in Xenopus laevis. Efforts have focussed on two of these, XLPOU1 and XLPOU2. Both are expressed as early as the neural plate - neurula stage in embryos. In adults, XLPOU1 is expressed only in skin and brain while XLPOU2 is seen in kidney and brain. In embryos, both are expressed in the dorsoanterior portion of the early tailbud stage and, consistent with this localization, are greatly reduced by UV treatment. In situ hybridization studies showed XLPOU1 transcripts to be localized in the anterior region of the neural plate and nerve cord. In the tailbud stage, they are found mostly in brain and eye, and there is a sharp anterior boundary beyond which expression does not occur. Because of the localization of XLPOU1 and XLPOU2 and their early expression, it appears that these genes may play an important role in the specification of neural phenotypes (Agarwal, James, Sato).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 45000-24 CEB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Thyroxine-Protein Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Robbins	Chief	CEB, NIDDK
Others:	H.J. Cahnmann	Scientist Emeritus	CEB, NIDDK

COOPERATING UNITS *(if any)*
Diane Rader (HNLBI), Salvatore Benvenga (University of Messina, Italy)

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Hormone Metabolism and Action Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have shown that the gel filtration profile of ^{125}I-T4 (T4) equilibrated with the total lipoprotein fraction from normal human plasma separates several HDL particles which bind T4. Most of the T4 is recovered in the smaller size HDL subfraction (corresponding to HDL_3). We also have shown that T4 binding to HDL is mediated by apolipoproteins, especially apoA-I. We have continued to characterize T4 binding to HDL subclasses by photoaffinity labeling with 0.5nM T4. Four preparations of HDL_2 and five of HDL_3 were labeled in the presence or absence of excess T4 or inhibitors of T4 binding to plasma proteins. Labeled HDL was anlyzed by SDS-PAGE and radioactivity associated with apoA-I quantitated. The results showed that HDL_3-apoA-I binds T4 more avidly and more consistently than HDL_2-apoA-I. Inhibition of T4 binding was also variable and was generally more effective with HDL_2. This suggests that the apoA-I in the two major HDL subclasses exists in different conformations having different abilities to bind T4.

In further studies we find that photoaffinity labeling (PAL) of intact HDL with T4 results in the labeling of several other apos including apo(a), apoB100, apoA-II, apoA-IV and apoE.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45004-20 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Structure of Polypeptide and Protein Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. Prasad	Visiting Associate	CEB, NIDDK
Others:	R. Lippoldt	Health Services Officer	CEB, NIDDK

COOPERATING UNITS (if any)

Washington University School of Medicine, St. Louis, MO (Dr. J. Heuser); Temple University School of Medicine, Philadelphia, PA (Dr. J.H. Keen)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Protein Structure Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been discontinued.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—854-938

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 45009-24 CEB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Studies of Thyroid Diseases

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	J. Robbins	Chief	CEB, NIDDK
Others:	K. Ain	Medical Staff Fellow	CEB, NIDDK
	D. Garcia	Medical Staff Fellow	CEB, NIDDK
	T. SHiver	Medical Staff Fellow	CEB, NIDDK
	M. Phyillaier	Biologist	CEB, NIDDK

COOPERATING UNITS (*if any*)
Dr. J. Norton, Surgery Branch, NCI; Dr. J. Reynolds, Nuclear Medicine, CC; M. Merino, Laboratory of Pathology, NCI; C. Meyers, NCI.

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Hormone Metabolism and Action Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Two forms of adjuvant therapy designed to improve the effectiveness of I-131 radiation in thyroid cancer are under study. The first is lithium ion, which is used to prolong the retention of I-131 in the cancer. Preliminary findings have been described in previous annual reports. The second is the use of doxorubicin (adriamycin) as a radiosensitizing agent. To evaluate its effectiveness, we have begun a randomized study of high-risk thyroid cancer patients who receive standard I-131 therapy with or without the administration of low-dose adriamycin ($10 mg/m^2$) 30 min preceding each I-131 treatment dose. To our knowledge, this is the first attempt to randomize patients in a therapy protocol for thyroid cancer employing I-131.

Other patients with negative I-131 whole body scans but elevated concentration of thyroglobulin in blood are given I-131 therapy in an attempt to localize the cancer by post-therapy scanning. Most patients have been found to have positive post-therapy scans and they are under evaluation for possible beneficial effects of the therapy.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1981 0—864-938

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45014-20 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Membranes and Secretion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	J. Wolff	Associate Chief	CEB, NIDDK
Others:	D. Sackett	Expert	CEB, NIDDK
	L. Knipling	Technician	CEB, NIDDK

COOPERATING UNITS *(if any)*

T. Shiver, NICHD

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Studies on the role of the cytoskeleton in steroidogenesis have been extended to intermediate filaments by use of acrylamide which collapses these filaments. Acrylamide treatment leads to marked cell rounding of Y-1 mouse adrenal cells in culture (like other stimulators of adrenal cells). There is stimulation of steroidogenesis which is slower than with ACTH or forskolin but the final extent of steroid production is similar. However, acrylamide, raises steroid output without changes in production of cAMP and thus appears to stimulate steroidogenesis by a different pathway.

U S GOVERNMENT PRINTING OFFICE 1991 0—[illegible]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45016-21 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Thyroid Hormone Secretion and the Function of Microtubules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Wolff	Associate Chief	CEB, NIDDK
Others:	D. Sackett	Senior Staff Fellow	CEB, NIDDK
	L. Knipling	Technician	CEB, NIDDK
	G. Obi	Guest Worker	CEB, NIDDK
	M. Bifulco	Guest Worker	CEB, NIDDK
	C. Laezza	Guest Worker	CEB, NIDDK

COOPERATING UNITS *(if any)*

Jay Knutsen, NHLBI
J. Thompson, NIDR

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.7	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of the domain structure of rat brain tubulin has revealed the following domains:

1. Larger N-terminal and smaller C-terminal domain in both α and β tubulin. These remain attached in the dimer under native conditions despite single bond cleavage in each monomer and microtubules can be formed from the split material. Intradimer contacts are β-C terminal domain to α-N terminal domain and the interdimer contacts in the microtubule are α-C terminal to β-N terminal.

2. The equilibrium association between the α and β monomers has been studied by two indirect means: a) exposure for proteolysis and b) binding of the flouresent dye, mile red, at the α/β interface, and directly by equilibrium certifugation with a new short column method. Dissociation constants vary from 0.2 - 1.0μM.

3. Thermodynamic analysis of dimerization by equilibrium ultracentrifugation reveals that monomer association is entropy-driven, is hydrophobic, and is sensitive to small ligands such as cholchine and GTP.

4. The colchicine binding site has been localized to the β-monomer by direct photolabeling. It appears to be near enough the α-submit so that the latter may contribute to binding.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-938

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45018-16 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenylate Cyclase and Other Extracellular Products of B. Pertussis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Wolff	Associate Chief	CEB, NIDDK
Others:	L. Knipling	Technician	CEB, NIDDK
	D. Sackett	Expert	CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is inactive.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—884-339

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45020-15 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthesis of Thyroxine Transport Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Robbins	Chief	CEB, NIDDK
Others:	L. Bartalena	Visiting Associate	CEB, NIDDK
	M. Phyillaier	Biologist	CEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.5	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The effects of interleukin-6 (IL-6) on the liver synthesis of thyroid-hormone binding proteins was investigated by biosynthetic labeling of human hepatoblastoma-derived Hep G2 cells using [35-S] methionine, followed by immunoprecipitation of media with specific antisera, and fluorography of immunoprecipitates. Slot blot hybridization of mRNAs to specific cDNAs was also carried out.

IL-6 produced a dose and time-dependent inhibition of T4 binding globulin, albumin and transthyretin. Similar effects were exerted on corticosteroid-binding globulin. On the other hand, IL-6 increased alpha 1-antitrypsin and alpha 1-antichymotrypsin synthesis. These effects appeared to occur mainly at a pretranslational level. The activity of IL-6 could be inhibited by preincubation with neutralizing anti-IL-6 antiserum, and could be reverted by removing the cytokine from the culture medium.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-930

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 45028-13 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Thyroid Hormone-Cell Interactions

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	J. Robbins	Chief	CEB, NIDDK

COOPERATING UNITS (*if any*)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Inactive, with intent to pursue studies later on the influence of lipoproteins on the entry of thyroid hormone into cells.

Publications

1. Lakshmanan M, Goncalves E, Lessly G, Foti D, Robbins J. The transport of thyroxine into mouse neuroblastoma cells, NB41A3: The effect of L-system amino acids. Endocrinology 126, 3245-3250, 1990.

2. Goncalves E, Lakshmanan M, Cahnmann HJ, Robbins J. High affinity binding of thyroid hormones to neuroblastoma plasma membranes. Biochim Biophys Acta 1055, 151-156, 1990.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-930

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45033-08 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Mapping of Triiodothyronine Responsive Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Nikodem	Senior Scientist	CEB, NIDDK
Others:	K. Petty	Research Fellow	CEB, NIDDK
	B. Desvergne	Visiting Fellow	CEB, NIDDK
	A. Raptis	Visiting Associate	CEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and ACtion Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. The CACCC box is a DNA sequence that functions as a positive transcriptional regulatory element in the promoters of several genes. The rat malic enzyme (ME) gene promoter contains two CACCC boxes at a site containing a tandem repeat of the sequence CTCGCCACCC (malic promoter element 2 or MPE2, -74/-48) which functions as a cis-regulatory element. A sequence similar to a half-site of MPE2is found in the human immunodeficiency virus long terminal repeat (HIV LTR, -67/-42) and the human c-fos promoter also contains a sequence identified as a retinoblastoma control element (RCE, -100/-70) which is similar to MPE2. We have now obtained two proteins binding to this site: 1. By screening a rat liver cDNA expression library with the MEP2 binding site and 2. using HeMEP2 DNA as an affinity matrix in purifying a protein from a rat liver nuclear extracts. Both proteins bind specifically to this site. However they also interact specifically with the malic enzyme thyroid hormone response element. Thus the same or similar nuclear proteins bind to two seperate transcriptional cis-regulatory elements in the ME gene promoter one of which also binds the T3 receptor. These liver nuclear proteins may play important roles in the ME gene transcriptional reponse to thyroid hormone. Further characterization of these proteins and DNA motifs to which they bind is being investigated.

2. The α and β receptors are highly related to each other, bind the same ligand, and to the same TREs, but they give quantitatively different responses when activated by hormone suggesting that they do not have redundant functions. The T3 receptor α variant protein exerts distinct effects on transactivating functions of these receptors by a mechanism that is still unclear.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-930

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 45034-08 CEB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
Regulation of Specific Rat Liver mRNAs by Thyroid Hormone

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	V. Nikodem	CEB, NIDDK
Others:	R. Lippoldt	CEB, NIDDK
	J.E. Rall	CEB, NIDDK
	M.K. Song	CEB, NIDDK
	D. Grieco	CEB, NIDDK

COOPERATING UNITS *(if any)*
Dr. S.M. Aloj and Dr. L. Kohn, LBM, NIDDK

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Hormone Metabolism and Action Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been kept in abeyance this year pending the results of other projects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45038-04 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Thyroid Hormone Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Nikodem	Senior Scientist	CEB, NIDDK
Others:	T. Mitsuhashi	Visiting Associate	CEB, NIDDK
	P. Hallenbeck	NRC Fellow	CEB, NIDDK
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	M. Phyillaier	Technician	CEB, NIDDK
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COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. The molecular basis of T3 dependent gene activation as studied utilizing nuclear extracts prepared from Spodoptera frugiperda cells infected with recombinant baculovirus overexpressing rat thyroid hormone receptor (rTRα) and H2RIIBP.
We showed that rTRα homodimerizes in solution, binds the hormone and both liganded and unliganded receptor homodimer interacts with an 18 bp region of the promoter of the malic enzyme gene (ME-TRE) as a homodimer. Furthermore the receptor formed heterodimers with either in solution or on the TRE, H-2RIIBP a non-hormone binding member of the steroid hormone receptor superfamily, when present in nuclear extracts prepared from Sf9 cells infected with a recombinant baculovirus overexpressing H-2RIIBP. Heterodimerization occurred at a higher rate than did homodimerization of either rTRα or H-2RIIBP. Heterodimerization was an important component of rTRα binding to TH and the ME-TRE since both were increased several fold when rTRα was allowed to heterodimerize with H-2RIIBP. Quantitative analysis of TH binding not only demonstrated the ability of the heterodimer to bind TH but also revealed that the heterodimer bound the same amount of TH as the homodimer did, suggesting a stoichiometry of 1 ligand per dimeric species. Interestingly, a heterodimer of rTRα and a rat liver nuclear protein was incapable of binding TH. Finally, the functional data demonstrated that cotransfection of plasmids expressing H-2RIIBP and rTRα increased the TH inducibility of ME-TRE over that observed with the rTRα alone. These results imply that thyroid hormone inducibility depends on the nature of rTRα dimeric species which in turn affects interaction with TREs.

2. We analyzed and determined sequences in the thyroid hormone receptor gene which are required for the promoter activity of this gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45040-03 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of Thyroid Hormone on Synthesis of Myelin Basic Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	V. Nikodem	Senior Scientist	CEB, NIDDK
Others:	A. Farsetti	Visiting Fellow	CEB, NIDDK
	B. Desvergne	Visiting Fellow	CEB, NIDDK
	J. Robbins	Chief	CEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.8	0.2

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using RNase protection analysis, we have quantitatively evaluated the levels of MBP mRNA isoforms in vivo during and after development in different thyroidal conditions. Our results showed that the hormone is not necessary for the developmentally programmed appearance of MBP mRNA in mice but is required for optimal MBP gene expression and that thyroxine treatment reverses the effect of hypothyroidism in young rats. Nuclear run-off assay showed that T3 increases the rate of transcription of the MBP gene. We further demonstrated that rat thyroid hormone receptor α specifically interacts with a ^{32}P-MBP gene fragment in the 5' flanking region (-256/+1) footprinting the region -186/-163. Moreover, the chimeric genes either -1317 MBP or -256 MBP : CAT confer thyroid hormone responsiveness when cotransfected with the receptor into NIH3T3 or NG108-15 cells.

Thus our results demonstrate that the mechanism underlying thyroid hormone regulation of MBP mRNAs is at least in part at the level of transcription, mediated by a thyroid hormone response element residing within 256 bp of the 5' flanking region of the MBP gene to which the liganded receptor binds.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-308

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45041-02 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of XLPOU 1 and XLPOU 2 in Xenopus Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Sato	Senior Staff Fellow	CEB, NIDDK
Others:	V. Agarwal	Visiting Fellow	CEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Endocrinology Branch

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5		

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  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Several members of the POU domain gene family have been shown to be important in tissue specific gene regualtion during development. We have cloned two DNA's encoding two unique Xenopus laevis POU-domain protiens, XLPOU 1 and XLPOU2, of the POU III protein class. Both genes are expressed as early as the neutral plate-neurula stage in embyros. In adults, XLPOU 1 expression is restricted to the skin and brain, while XLPOU 2 expression is observed in kidney and brain. In dissected embryos, both XLPOU 1 and XPLOU 2 are expressed in the dorsoanterior portion of the early tailbud embryo. Consistent with this localization, UV treatment, a condition that "posteriorizes" embryos, greatly reduces the expression of XLPOU 1 and XLPOU 2. In situ hybridization studies show that XLPOU 1 transcripts are localized in the anterior region of the neutral plate and nerve cord. In tailbud staged embryos, the XLPOU 1 transcripts are localized mostly in the brain and eye. There is a sharp anterior boundary of expression; the most anterior region of the brain does not hybridize. The posterior boundary of expression, apparent in tailbud stage embryos, is at the junction of the hindbrain and spinal cord. We believe that XLPOU 1 and XLPOU 2, because of their localized and early expression in embryos may play an important role in the specification of neuronal phenotypes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 45042-02 CEB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of Xenopus-posterior (Xpo) in Early Frog Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Sato	Senior Staff Fellow	CEB, NIDDK

COOPERATING UNITS *(if any)*

Dr. Tom Sargent, Laboratory of Molecular Genetics, NICHD
Dr. Christine Holt, Department of Biology, UCSD

LAB/BRANCH

Clinical Endocrinology Branch

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Classical embryology studies in amphibians have demonstrated that important step in anterpopsterior axis formation is the polarization of the prospective mesoderm. The dorsal mesoderm then transfers this polarity by induction to the overlying ectoderm during gartrulation. The molecular nature of the mesodermal polarity is not known although a number of specific gene products, such as the homeobox gene, Xhox 3, are thought to play a role. We have isolated and characterized another potential regulator of anteroposterior axis formation, Xenopus-posterior (Xpo). The Xpo protein belongs to a new class of developmentally expressed proteins. It is activated at mid-blastula transition and is expressed maximally during mid-gastrulation to neurulation . Its expression is restricted to the posterior dorsal mesoderm and ectoderm. A single copy of a finger motif, of the "CCHC" type, is located in the carboxyl terminus. Related motifs are found in the nucleocapsid protein of all retroviruses where this domain serves an essential role in retroviruses where this domain serves an essential role in retroviral replication and in cellular nucleic acid binding protein (CNBP), a protein that binds to the sterol regulatory element. As with other potential regulators of axis formation, Xpo expression is induced in ectodermal explants by treatment with basic FGF and polypeptide growth factors found in the medium conditioned by the Xenopus XTC cell line. Taken together, Xpo's properties suggest that it may play a role in the organization of the anteroposterior axis during development.

U.S. GOVERNMENT PRINTING OFFICE 1991 O— 4 939

ANNUAL REPORT OF THE LABORATORY OF THE DIABETES BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

Investigators in the Diabetes Branch conduct clinical investigation and basic scientific research with special emphasis on understanding the mechanism of action of insulin and the causes of diabetes mellitus. Towards this end, a multi-disciplinary approach has been applied, involving techniques of molecular biology, cell biology, biochemistry, and clinical physiology. Other projects include studies of hormones and other messenger molecules important to the regulation of growth and development, especially growth hormone and insulin-like growth factors I and II. In addition, there is an active research program with respect to acromegaly, a clinical disease caused by the overproduction of growth hormone.

Recognition of previous achievements

The contributions of investigators in the Diabetes Branch have been recognized in many ways. First, several investigators have received research support from private funding agencies: the Juvenile Diabetes Foundation, the American Diabetes Association, and the Diabetes Research and Education Foundation. Second, Dr. Phillip Gorden serves as Director of the NIDDK. Third, investigators in the Diabetes Branch serve on the Editorial Board of several internationally respected journals. Fourth, the senior members of the Diabetes Branch receive frequent invitations to lecture at national and international meetings. Fifth, over the years several individuals in the Branch have been recognized by receiving awards for their research.

The insulin gene and its regulation of expression

Insulin is a hormone synthesized predominantly in the β-cell of the pancreas. In addition, investigators in the Diabetes Branch have presented evidence that the hormone is synthesized in other locations as well. To pursue investigations of the regulation of expression of the insulin gene in both the pancreas and elsewhere, recent studies have employed the species Xenopus laevis, an important experimental model system to study early development. Two non-allelic genes for insulin have been identified in this species, and the cDNAs encoding these two insulins have been cloned. A new technique involving the polymerase chain reaction has been developed to amplify insulin cDNA. This new technique has been used to detect minute quantities of insulin mRNA even in unfertilized Xenopus oocytes. The insulin mRNA is of maternal origin, and disappears shortly after fertilization of the egg. During embryonic development, transcription of the insulin gene begins again shortly after neurulation. At this time, insulin mRNA localizes primarily in the most cephalic regions. This raises the possibility that insulin may play a role in the development of the central nervous system.

Insulin is the hormone that is the most important to regulation of glucose metabolism. Defects in insulin secretion can cause diabetes. For the most part, at the time individuals are diabetic, their insulin levels tend to be low. However, in patients destined to develop noninsulin-dependent diabetes, hyperinsulinemia is frequently present prior to the development of diabetes. To evaluate whether hyperinsulinemia may play a role in causing insulin resistance in diabetes, a transgenic mouse model has been developed. In this transgenic mouse, the human insulin gene is overexpressed in the β-cell. Thus, these mice are hyperinsulinemic because of the simultaneous expression of both murine and human insulin. The degree of hyperinsulinemia in these transgenic mice correlates with the number of copies of the human insulin gene integrated into the genomic DNA of the mice. These mice develop mild insulin resistance and also hyperglycemia, suggesting that primary hyperinsulinemia can lead to glucose intolerance and insulin resistance, both abnormalities typical of noninsulin-dependent diabetes.

Other studies have been carried out directly in human subjects. The possibility that mutations in the insulin gene may play a role in the development of the common form of noninsulin-dependent diabetes mellitus has been evaluated by determining the nucleotide sequence of the insulin gene in several patients with noninsulin-dependent diabetes mellitus, including Pima Indians and Nauruans, two ethnic groups with a high prevalence of diabetes. In all cases, the sequence of the insulin gene appears to be normal. Therefore, no evidence could be found that mutations in the insulin gene contributed to the pathogenesis of diabetes in these patients.

Insulin receptors

The insulin receptor is a heterotetrameric glycoprotein located on the cell surface. It is encoded by a single gene that encodes a precursor molecule. The precursor undergoes multiple post-translational processing steps including proteolytic cleavage to yield two different types of subunits: an α-subunit that contains the insulin binding site and a β-subunit that is a tyrosine specific protein kinase. When insulin binds to the extracellular domain of the receptor, this activates the tyrosine kinase associated with the intracellular domain of the receptor. Activation of the tyrosine kinase plays a necessary role in mediating insulin action. Several projects in the Diabetes Branch pertain to various aspects of insulin receptor biosynthesis and insulin receptor function.

To study the regulation of the expression of the insulin receptor gene, the DNA in the 5' flanking region of the human insulin receptor gene has been cloned. Deletion analysis has been employed to identify a 70-base pair region of the gene that contains the majority of the promoter activity. In addition, a weak enhancer has been identified upstream of the promoter. Further investigations are directed towards identifying other regulatory domains that may play a role in the developmental and endocrine regulation of the expression of the insulin receptor gene. For example, there are three consensus binding sites for the transcription factor C/EBP. Expression vectors containing these sequences can be transactivated by an expression vector for the transcription factor C/EBP. The role of C/EBP in the 10-fold induction of the insulin receptor gene during adipocyte differentiation is being investigated. This induction seems to require a portion of the intron that contains one of the C/EBP binding sites.

In another project, several steps involved in post-translational processing of the insulin receptor during biosynthesis are being investigated. In particular, N-linked glycosylation, O-linked glycosylation, and fatty acylation are being studied. The role of N-linked glycosylation is being investigated by site-directed mutagenesis to eliminate the N-linked glycosylation sites. By expression of the cDNAs encoding these mutant receptors, it may be possible to determine the functional role of the N-linked glycosylation. Progress has also been made toward identifying the O-linked glycosylation site in the β subunit.

The role of the tyrosine kinase in mediating insulin action is also being investigated. A 120 kilodalton glycoprotein in rat liver membranes has been identified to serve as a substrate for phosphorylation by the insulin receptor. This glycoprotein has been immunoaffinity purified, a partial amino acid sequence has been determined, and a cDNA encoding this glycoprotein has been obtained. This protein substrate, pp120, appears to be identical to a previously identified enzyme that has the property of hydrolyzing ATP and GTP. The effect of phosphorylation to regulate the enzymatic activity of this protein is presently being investigated. By expressing the cDNA through transfection, it should be possible to determine the physiological role of this protein, and what role the protein may have in mediating the effect of insulin upon target cells.

In addition, as part of a long term collaboration with scientists at the University of Geneva, studies are underway to explore the mechanism and significance of receptor-mediated endocytosis, receptor internalization, and recycling.

Considerable progress has been made in identifying mutations in the insulin receptor gene, and elucidating the role of this type of genetic defect in causing human disease. Multiple different mutations have been identified in the insulin receptor gene, these fall into five classes: class 1, mutations that inhibit insulin receptor biosynthesis, frequently by decreasing levels of insulin receptor mRNA; class 2, mutations that impair the transport of mutant receptors to the cell surface; class 3, mutations that decrease the affinity with which insulin is bound to the receptor; class 4, mutations that impair tyrosine kinase activity; class 5, mutations that accelerate receptor degradation, apparently by inhibiting recycling of internalized receptors back to the plasma membrane. Most of these mutations have been identified in patients with relatively rare genetic syndromes associated with severe insulin resistance and acanthosis nigricans. At present, studies are underway to determine the prevalence of this type of mutation, and also to investigate the possibility that this type of mutation may contribute to the pathogenesis of more common diseases such as noninsulin-dependent diabetes mellitus and polycystic overary disease.

One allele of the insulin receptor gene in cultured 3T3-L1 cells has been inactivated by the technique of homologous recombination. Inactivation of the insulin receptor gene was associated with a marked impairment in the ability of the 3T3-L1 cells to differentiate into adipocytes in vitro. This suggests an important role for the insulin receptor in regulating the development and differentiation of fat cells. The method of homologous recombination is now being applied in an effort to inactivate the insulin receptor gene in embryonic stem cells. Eventually, this approach should enable the production of transgenic mice with mutations in the insulin receptor gene and also other genes relevant for glucose homeostasis and diabetes. The production of animal models of disease by this technique should be very useful in studying both the causes and treatment of diabetes.

Insulin-like growth factors

Previously, cDNAs encoding IGF-I have been cloned from rat liver. In addition, part of the rat IGF-I gene has been cloned. More recently, two non-allelic IGF-I genes have been cloned from the species Xenopus laevis. Regulation of the expression of the rat IGF-I gene has been studied extensively. This gene, subsequent to transcription, undergoes a complex pattern of splicing. There are at least three 5'- untranslated region sequences, the steady-state levels of which are differentially regulated in vivo by growth hormone in a tissue-specific manner. In addition, there are two alternative splicing patterns of the 3'-end that result in two different propeptides being encoded. The heterogeneity of IGF-I mRNA results from both differential splicing and utilization of alternate polyadenylation sites. The physiological significance of these alternative splicing patterns is presently being investigated.

IGF-I gene expression in the liver increases dramatically postnally and especially around puberty. These changes are reflected in the marked rise in serum IGF-I levels. In extrahepatic tissues, e.g., the brain, IGF-I is also expressed prenatally and follows a specific developmental pattern. Prenatally, mesenchymal tissues are the primary source of IGF-I, whereas, postnatally, parenchymal cells, e.g., neuronal cells, express IGF-I. Thus, prenatally in the nervous system, IGF-I may act as a developmental growth factor and postnatally may play a role in neurotransmission, or as a neurotrophic factor.

In the ovary, as with most extrahepatic tissues, IGF-I gene expression is less affected by growth hormone (GH). Indeed, estrogen stimulates IGF-I gene expression, as measured by steady-state levels of IGF-I mRNA. Since IGF-I stimulates steroidogenesis and is expressed by granulosa cells, it may obviously play an important role in ovarian physiology, presumably through local autocrine and paracrine mechanisms.

Unilateral nephrectomy results in compensatory enlargement of the remaining kidney. In adult rats this primarily induces hypertrophy of existing cells and is regulated by GH, since isolated GH deficiency prevents the hypertrophy and no increase in IGF-I gene expression is seen, suggesting that the GH effect may either be direct or via elevated systemic IGF-I levels. In contrast, compensatory hypertrophy in immature animals primarily involves hyperplasia and is associated with increased kidney IGF-I gene expression. This increased expression is not GH-dependent and is probably causative in the hyperplastic process.

Thus, the IGFs play an important role in normal development and certain pathological states, and future studies will hopefully elucidate the molecular mechanisms underlying these diverse processes involving IGF-I action.

During postnatal life, the principal recognized role of IGF-I is to promote growth. The possibility that IGF-I has a role in regulating embryonic development is being investigated using the chicken embryo system. IGF-I can be detected in the yolk of the fertilized chicken egg. Moreover, the chicken IGF-I gene is expressed in the blastoderm and the level of expression increases during organogenesis. The regulation of the expression of the IGF-I gene differs in different tissues. For example, in brain and pancreas, IGF-I is expressed during mid-embryogenesis while in the liver its expression is only significant after hatching. The mechanism whereby IGF-I regulates differentiation and development is being examined using the lens of the eye. In the lens, IGF-I stimulates the expression of the gene encoding δ-crystallin. At present, the cis-acting elements and trans-acting factors that mediate the regulation of δ-crystallin by IGF-I are being mapped. The effects of IGF-I to regulate transcription have also been studied in another system. In cultured neuronal and glial cells, IGF-I, insulin, and phorbol esters all increased the expression of a gene encoding the rat brain/HepG2 glucose transporter (GLUT 1).

Insulin-like growth factor receptors

The biological effects of IGF-I are mediated through a cell surface receptor, a heterotetromeric glycoprotein that is homologous to the insulin receptor. A cDNA encoding a portion of the IGF-I receptor has been cloned from rat granulosa cells. In addition, partial length cDNAs encoding receptors in this family have been cloned from Xenopus embryos. A portion of the 5' flanking region of the IGF-I receptor gene has been cloned from the rat. The promoter region of the gene has been identified. This region contains a unique transcription initiator sequence with a single start site, previously found in a number of developmentally regulated genes. The promoter region lacks a TATA box, but contains putative binding sites for several transcription factors: Sp1, ETF, GCF, and AP2.

The expression of the IGF-I receptor gene is regulated under many physiological and pathophysiological conditions. The developmental regulation of IGF-I receptor gene expression demonstrated tissue-specific changes; in most tissues there was a dramatic postnatal decrease. Brain demonstrated the highest prenatal levels of receptor gene expression.

Insulinopenic diabetes is associated with early changes in renal function. These include enlargement of the kidneys and elevated levels of glomerular filtration rate and renal plasma flow. Both GH and IGF-I can cause these changes, yet insulinopenic diabetes is often associated with reduced serum GH and IGF-I levels in the rat. In contrast, the binding of IGF-I and IGF-II to kidney membranes was increased, under these conditions. Using antisense RNA probes for these receptors, we have shown that expression of the receptor gene is indeed elevated within 24 hours of induction of insulinopenic (streptozotocin-induced) diabetes. This elevated receptor level may also contribute to the early renal changes in diabetes.

Compensatory renal enlargement rapidly follows unilateral nephrectomy. In immature rats, the enlargement of the contralateral kidney is primarily hyperplastic and is associated with increased steady-state levels of IGF-I receptor mRNA as well as increased IGF-I binding to kidney membranes. In contrast, in adult rats, the enlargement is associated with hypertrophy and constant IGF-I receptor mRNA levels, suggesting that the IGF-I receptor mediates hyperplasia in immature rats but is not involved in the hypertrophy characteristics of adult animals.

Growth hormone and acromegaly

A long term follow-up study of patients with acromegaly has been conducted in the Diabetes Branch over the past 25 years. Several modes of therapy have been employed, including radiation therapy, trans-sphenoidal hypophysectomy, and more recently treatment with drugs such bromocryptine and somatostatin analogs. Recent studies have demonstrated that somatostatin analogs can be extremely useful in lowering growth hormone levels in patients with acromegaly. In addition, this agent can be useful in reducing hormonal hypersecretion caused by tumors of the pituitary that secrete thyroid stimulating hormones (TSH) and also in glucagon secreting tumors of the pancreas. Unfortunately, somatostatin analogs have a side effect of causing the patients to develop thickened bile. Because of the previous observation that patients with somatostatin secreting tumors have a high prevalence of gallstones, it seemed important to determine how frequently treatment with somatostatin analogs would also cause this complication.

Of patients who have received long-term treatment with the long-acting somatostatin analog, 70% of the patients with acromegaly have developed gallbladder sludge and 20% have deveveloped gallstones. Patients currently enrolled in this study who have persistent sludge or gallstones will be treated with ursodeoxycholate, a drug with the potential to prevent or to reverse the formation of sludge and gallstones.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47001-10 DB

PERIOD COVERED

October 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Phosphorylation of Insulin and IGF-I Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	C.T. Roberts, Jr.	Research Biologist	DB/NIDDK
Others:	H. Werner	Visiting Associate	DB/NIDDK
	B. Stannard	Biologist	DB/NIDDK
	M. Adamo	Staff Fellow	DB/NIDDK
	H. Kato	Visiting Fellow	DB/NIDDK

COOPERATING UNITS *(if any)*

University of Florida (M. Raizada)
Dept. of Physiology

LAB/BRANCH

Diabetes Branch

SECTION

Section of Molecular and Cellular Physiology

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Summary

Insulin and IGF-I receptors are the products of distinct genes and mediate different functions. The insulin receptor regulates metabolic functions whereas the IGF-I receptor regulates mitogenic functions. Both receptors possess tyrosine kinase activity, but exhibit distinct tissue specificities, development regulation and certain structural differences. Furthermore, in tissues such as the brain, the insulin and IGF-I receptors differ from their counterparts in other tissues. To investigate these differences, we have cloned and sequenced the rat IGF-I receptor cDNA and its proximal promoter region. These studies as well as investigations of IGF-I receptor gene expression in development and certain pathological states will contribute to our understanding of the precise mechanisms and functions of the IGF-I receptor. In addition, cDNA probes for various glucose transport proteins are used to study the role of insulin and IGF-I in the metabolic functions of brain tissue which express large numbers of IGF-I receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z0I DK 47002 - 04 DB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)
Insulin Gene Expression and Insulin Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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	A. Shuldiner	Senior Staff Fellow	A. Bodner	Guest Worker	DB/NIDDK
	L. Marban	Guest Worker	K. Tanner	Spec. Vol.	DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	6.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Molecular biological techniques are being used to discern gene expression of insulin, insulin-like growth factors (IGFs) and their receptors and actions in several vertebrate model systems. Insulin is apparently a requirement for normal development, and the chicken embryo is one of the suitable models for studying the role of insulin in development. The ontogeny of expression of the insulin gene has been studied and the gene for IGF-I and IGF-I receptor are cloned. The role of insulin in cell differentiation and gene expression in the eye lens of chick embryo is also being used as a cell model to understand the action of insulin in development. The amphibian, Xenopus laevis, is also a model system used to study development. The amphibian insulin and insulin-like growth factor I genes have been isolated and their sequences determined and confirmed by molecular cloning. Studies are in progress to define the expression of these peptides during Xenopus development using the polymerase chain reaction.

A transgenic mouse line with multiple copies of the human insulin gene integrated into its genome has been established. The degree of hyperinsulinemia correlates with human gene copy numbers. The transgenic mice provide a model system for studies in regulation of insulin gene expression and the effects of chronic hyperinsulinemia on glucose homeostatis.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47005 - 19 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Insulin Receptors in Circulating cells in man

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	P. Gorden	Director	NIDDK
Others:	C. Hendricks	Biol. Lab Tech.	DB/NIDDK
	E. Collier	Expert	DB/NIDDK
	P. Roach	Clinicl Associate	DB/NIDDK
	S.I. Taylor	Chief	DB/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The present work continues prior investigations of insulin receptors on circulating cells in patients with insulin resistance and diabetes mellitus. Insulin receptors are evaluated for their ability to bind insulin and to act as tyrosine-specific protein kinases. We are also investigating circulating anti-insulin receptor antibodies from patients with Type B extreme insulin resistance for their ability to stimulate various insulin actions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-DK-47007-15 DB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (*80 characters or less Title must fit on one line between the borders.*)
Antibodies to Receptors

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)
S. Taylor, Chief Diabetes Branch

COOPERATING UNITS (*if any*)

LAB/BRANCH
Diabetes Branch/ Section Biochemistry & Molecular Pathogenesis Section

SECTION
Biochemistry & Molecular Pathogenesis Section

INSTITUTE AND LOCATION
NIDDK

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Project is inactive

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 47009 - 04 DB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Positron Emission Tomography/NMR Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	R. Eastman	Clinical Director,	DB/NIDDK
Others:	S. Taylor	Chief,	DB/NIDDK
	D. LeRoith	Section Chief,	DB/NIDDK
	C. Cochran	RN,	CC

COOPERATING UNITS *(if any)*
Richard Carson, Nuclear Medicine; Clifford Bogardis - NIH, Phoenix; Peter Butler, Anton Usala - E. Carolina Un.; Joseph Frank, Robert Balaban -- Diagnostic Radiology, CC; C. Phenekos - Athens, Greece.

LAB/BRANCH
Diabetes Branch

SECTION
Receptor and Hormone Action Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Protocols for project during 1991 are being developed.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 47014-21 DB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Acromegaly and Growth Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director	NIDDK
Others:	C.M. Hendricks	Bio. Lab Tech.	DB/NIDDK
	R.F. Arakaki	Senior Staff Fell.	DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Clinical and Cellular Biology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.0	OTHER: .05

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been incorporated in project Z01 DK 47027 - 06 DB.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47018 14DB

PERIOD COVERED

October 1, 1990 September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular Hormone-Like Peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	D. LeRoith	Section Chief	DB/NIDDK
	J. Roth	Director (DIR)	DB/NIDDK
Others:	C. T. Roberts	Research Biologist	DB/NIDDK
	M. Adamo	Staff Fellow	DB/NIDDK
	M. Bach	Med. Staff Fellow	DB/NIDDK
	H. Foyt	Med. Staff Fellow	DB/NIDDK
	A. Liotta	Expert	DB/NIDDK
	L. Sankaran	Chemist	DB/NIDDK

COOPERATING UNITS *(if any)*

University of Maryland, Dept. Obstetrics & Gynecology (E. Hernandez & E. Adashi)
Georgetown University, Dept. Physiology (A. Haramati and S. Mulroney)

LAB/BRANCH

Diabetes Branch

SECTION

Section of Molecular & Cellular Physiology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Summary

Hormone-like peptides including insulin, the insulin-like growth factors (IGF-I and IGF-II), somatostatin and ACTH are evolutionarily conserved and therefore have important roles in normal physiology. To further elucidate these roles, we have cloned and characterized multiple rat IGF-I cDNAs. Using these clones together with IGF-II cDNAs, we have studied the expression of these genes in normal development and in pathological states such as starvation, diabetes, and compensatory renal hypertrophy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47019 - 14 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Morphologic Studies of Ligand Binding to Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	P. Gorden	Director	NIDDK

COOPERATING UNITS *(if any)*

Institute of Histology and Embryology,
University of Geneva School of Medicine, Geneva, Switzerland.
(J.L. Carpentier, L. Orci) - Foreign

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This work represents over 13 years of collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of receptor-mediated endocytosis similar to other biologically important ligands that bind to cells. In the present study, using election microscopy, we find there is anatomical correlation between the dissociation of ^{125}I-insulin and its localization on the cell surface. This work has now been extended to include an insulin-resistant cell line that has an abnormal surface which leads to a higher association of ligand to the non-villous portion of the cell surface. Further, receptor-mediated endocytosis also appears to be regulated in hypoinsulinemic states. In both rat and in human type I diabetes there is an inhibition of ^{125}I-insulin internalization in the hyperglycemic state: the normal state is restored by insulin treatment. The role of intracellular calcium on the endocytotic process, as well as the relationship of stimulators of protein kinase C to internalization of both insulin and unrelated ligands such as transferrin, have been studied also. In addition, the function of the small non-coated invaginations in receptor-mediated endocytosis is being investigated.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47022 - 12 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Insulin Receptors in Syndromes of Extreme Insulin Resistance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.	S.I. Taylor	Chief, DB, NIDDK	M. Quon	SSF, DB	L. Ottini	Spec. Vol., DB
Others	A. Cama	Vis. Assoc, DB,	P. Gorden	Director,NIDDK	B. Koller	SSF, DB
	T. Kadowaki	Vis. Fell., DB	J. Roth	Dir.,DIR,NIDDK		
	H. Kadowaki	Spec. Vol., DB	E. Imano	Vis. Fell., DB		
	D. Accili	Spec. Vol., DB	R. L-Toledano	Vis. Fell., DB		
	C. Mckeon	Expert, DB	L. Sierra	Biologist, DB		
	M.A. Lesniak	Chemist, DB	F. Barbetti	Vis. Fell., DB		
	C.Hendricks	Biotech, DB	Nina Raben	Vis. Assoc., DB		

COOPERATING UNITS *(if any)*

Luitgard Mosthalf, Axel Ullrich - Max Planck Institute, Munich, Germany; Steve Lillioja - NIH, Phoenix; Kawamori R. - Osaka Un., Japan; G. Chrousos - NICHD; C. Bevins - Phila, PA; P. Zimmet - Victoria, Aust.; SW Serjeantson - Canberra, Aust.

LAB/BRANCH

Diabetes branch

SECTION

Biochemistry & Molecular Pathogenesis

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
10.0	8.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Insulin resistance contributes to the pathogenesis of several disease states including obesity and noninsulin-dependent diabetes mellitus (NIDDM). We have investigated the insulin receptor gene in patients with genetic forms of insulin resistance to gain insight into biochemical defects that give rise to disease.

Five classes of mutations have been identified:

1. Impaired receptor biosynthesis, due to either decreased levels of insulin receptor mRNA and/or premature chain termination mutations.
2. Impaired transport of receptors to the plasma membrane, due to missense mutations in the extracellular domain of the receptor.
3. Decreased affinity of insulin binding.
4. Decreased activity of the insulin receptor tyrosine kinase.
5. Accelerated receptor degradation associated with resistance to the effect of acid pH to dissociate insulin from its receptor within the endosome.

Studies are presently underway to estimate the prevalence of mutations in the insulin receptor gene in order to determine whether mutations in this gene contribute to the pathogenesis of the common form of noninsulin-dependent diabetes mellitus.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47024 - 12 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Biosynthetic Labeling of the Insulin Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	E. Collier	Expert	DB/NIDDK
Others:	H. Caro	Visiting Fellow	DB/NIDDK
	A. Ohali	Special Vol.	DB/NIDDK
	P. Gorden	Director	NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have studied the post translational modifications of the insulin receptor, i.e., glycosylation, fatty acylation, and phosphorylation using biosynthesis labeling of the insulin receptor. Based on the differential sensitivity of tryptic peptides biosynthetically labeled with radioactive sugars to endopeptidases, we have shown that the insulin receptor contains O-linked oligosaccharides. The peptide containing the O-linked oligosaccharides was localized to the amino terminal tryptic peptide of the beta subunit by specific immunoprecipitation with an anti-peptide antibody to the 12 amino acids at the amino terminus of the beta subunit. Using an inhibitor of O-linked glycosylation, benzyl-N-acetyl-galactosaminide, we confirmed the finding of O-linked oligosaccharides and investigated their function. In the presence of this inhibitor the binding of insulin to the insulin receptor and the autophosphorylation of the beta subunit was unchanged. The investigation of fatty acylation of the insulin receptor, previously reported by us in IM-9 lymphocytes, was extended to now show acylation of the receptor overexpressed in 3T3 fibroblasts transfected with the cDNA for the human insulin receptor. Both [^{3}H]myristic and [^{3}H]palmitic acid labeled the proreceptor and the mature subunits. The time course of labeling showed early labeling of the proreceptor and later appearance of label in the mature subunits. Thus, acylation of the insulin receptor is not limited to a single cell type.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 339

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47025 - 07 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Tissue Receptors for Insulin and Insulin-like Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	J. Roth	Director, DIR	DB/NIDDK
Others:	M.A. Lesniak	Chemist	DB/NIDDK
	M. Rojeski	Senior Staff Fellow	DB/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Diabetes Branch

SECTION

Receptor and Hormone Aciton Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD

TOTAL MAN-YEARS: 3.0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project Discontinued

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47026 - 07 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Tyrosine-specific protein kinase activity associated with the insulin receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.	Simeon Taylor	Chief	DB, NIDDK
Others:	Domenico Accili	Visiting Scientist	DB, NIDDK
	Sonia Najjar	IRTA	DB, NIDDK
	Alessandro Cama	Visiting Scientist	DB, NIDDK
	Keith Tanner	Spec. Volunteer	DB, NIDDK

COOPERATING UNITS *(if any)*

University of Catanzaro, Catanzaro, Italy (Nicola Perrotti)
Howard University, Washington, DC (S. Seminara)

LAB/BRANCH

Diabetes Branch

SECTION

Biochemistry & Molecular Pathogenesis

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

In the first step in insulin action, insulin binds to its receptor on the surface of the target cell. The insulin receptor is a transmembrane protein that possesses tyrosine-specific protein kinase activity. When insulin binds to the extracellular domain of the receptor, this activates the receptor tyrosine kinase activity. A growing body of evidence suggests that the activation of the receptor's tyrosine kinase is a necessary step in initiating the biological actions of insulin. Accordingly, we have embarked upon a search for intracellular proteins that are substrates for phosphorylation by the receptor-associated tyrosine kinase. We have identified one such substrate in rat liver plasma membranes: a glycoprotein with an apparent molecular weight of 120,000 (pp 120). In addition to being a substrate for the insulin receptor, pp120 can be phosphorylated by the receptors for epidermal growth factor and insulin-like growth factor I. pp 120 is present in liver from several species, but has not been identified in other tissues. The glycoprotein (pp120) was immunoaffinity-purified using monoclonal antibody HA4. Based on partial amino acid sequence data, pp120 has been tentatively identified as ectoATPase - an enzyme associated with hepatocyte plasma membranes. Studies are underway to express ectoATPase by transfection of cDNA in tissue culture cell lines. This will facilitate answering the question of whether tyrosine phosphorylation regulates the enzymatic activity of ectoATPase.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47027 - 06 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Use of SMS 201 - 995 in Hormone Secreting Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	R. Eastman	Clinical Director	DB/NIDDK
Others:	C.M. Hendricks	Bio. Lab Technician	DB/NIDDK
	P. Gorden	Director	NIDDK
	P. Roach	Clinical Associate	DB/NIDDK

COOPERATING UNITS *(if any)*

B. Weintraub Chief, MCNEB, NIDDK

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	.05

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to investigate the clinical usefulness of a long-acting somatostatin analogue (SMS 201-995) in controlling the signs and symptoms of hormone secretion by tumors. Patients who respond are offered long-term treatment and the effects of the analogue on clinical and biochemical tumor behavior is assessed. Of the patients who have been controlled to date, 70% of patients with acromegaly have developed gallbladder sludge and 20% have developed gallstones, and two patients with TSH secreting tumors have developed gallstones. Patients currently enrolled who have persistent sludge or stones will be treated in an open-label trial with ursodeoxycholate. New patients to the study will enter a randomized, placebo controlled trial of ursodeoxycholate (or placebo) plus SMS 201-995, to determine whether sludge and stone formation can be prevented. Ultrasound examinations of the gallbladder will be performed at baseline, 4-6 weeks, 3 months, and 6 months of the trial. If the agent proves useful for preventing sludge formation, we will offer patients continued treatment with ursodeoxycholate in an open label trial for up to two years.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47028-02 DB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Insulin Gene Expression and Insulin Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Catherine McKeon	Expert	DB, NIDDK
Assoc. Inv.	Simeon Taylor	Chief	DB, NIDDK
Others:	Tony Pham	Spec. Vol.	DB, NIDDK
	Gillian Walker	Spec. Vol.	DB, NIDDK
	Hui Chen	Spec. Vol.	DB, NIDDK
	Domenico Accili	Vis. Assoc.	DB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Diabetes Branch

SECTION

Biochemistry & Molecular Pathophysiology

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	6.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Regulation of the number of insulin receptors on the cell surface plays a critical role in determining insulin sensitivity. Previously, we have demonstrated that the insulin receptor is regulated at the transcriptional level by glucocorticoids. In order to study the mechanism of transcriptional regulation of the insulin receptor gene, we have cloned the 5' end of the human insulin receptor gene. We have begun to characterize the proximal promoter and find it has many features of a "housekeeping gene". We have localized a weak enhancer upstream of the promoter. This enhancer has a similar sequence to those found in other "housekeeping" genes. The proximal promoter is probably responsible for the low level expression of the insulin receptor gene which occurs in most cell types. Recently we have identified a region of the first intron which may be involved in tissue specific regulation. This region seems to be responsible for the 10-fold induction of the insulin receptor gene during adipocyte differentiation. Further studies to identify sequences responsible for the transcriptional regulation are underway.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

ANNUAL REPORT OF THE CLINICAL HEMATOLOGY BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

Study of Immunology of Blood Cell Deficiencies

Objectives:

To study the immunochemistry of allergic disorders affecting blood cells and effects of the immune reactions on cellular physiology, biochemistry, and *in vivo* cellular kinetics.

Methods employed:

Isoelectric focusing; anion exchange chromatography; enzyme activity assays; gel filtration of platelets; platelet activation by thrombin and other agonists; nitrocellulose and PVDF blotting for sequencing; microtiter enzyme-linked immunoassays for Ig quantitation; fluorescence-activated cell scanning; purification of antibodies by adsorption/elution; monoclonal antibody immobilization of platelet antigens (MAIPA) assays; fibrinogen binding of platelets; complement fixation; electron microscopy; native gel electrophoresis; enzyme (calpain) purification from platelets; cryoprecipitation; lectin chromatography for glycoprotein purification; immunoprecipitation; monocyte elutriation; protein and RNA synthesis using labeled precursors; specific mRNA assays for IL6, IL1, and TNF; intracellular Ca^{++} flux measurements; HPLC analysis of arachidonate metabolites, prostaglandins, leukotrienes, and HETEs.

1. Pathophysiology of Idiopathic Thrombocytopenic Purpura

Findings:

ITP is the most common acquired hemorrhagic disease and affects women 3 times more often than men. It is caused by immune destruction of platelets, but the nature of the immune injury is obscure. Macrophages appear to participate in destruction of platelets sensitized by antibodies as evidenced by therapeutic responses to splenectomy and adrenocorticosteroids within a few days with no discernible decrease in circulating antiplatelet antibody. We have developed a method to directly test macrophage participation in immunoreactions involving platelets.

We measured metabolic indicators of monocyte activation to determine objectively whether ITP antibodies activate monocytes in the presence of platelets. Elutriated, washed monocytes were sensitized (armed) for 1 hr at 4° and 37°C. with 95% pure IgG from ITP, post-transfusion purpura (PTP) or normal plasma, washed, and loaded with ^{3}H-arachidonic acid (AA) for 18 hr at 25°C. Platelets adherent to tissue culture wells after 18 hr incubation at 4°C and washing, were target cells to which armed monocytes were exposed for 18 hr at 37°C. Secreted AA metabolites in culture supernatants were analyzed by HPLC. Cytokines were measured by Northern blots of cell homogenates. Monocytes exposed to IgG from normal persons generated <0.5% AA equivalents (AAE) of leukotrienes (LT) or hydroxyeicosanoic acids (HETE). Monocytes exposed to 3 different anti-Pl^{A1} preparations generated 1-2% AAE of LT/HETE with Pl^{A1}(+) platelets, but <0.5% AAE with Pl^{A1}(-) platelets. Minimal IL1-beta and no significant tumor necrosis factor was produced with Pl^{A1}(+) platelets. Monocytes sensitized with IgG fractions from 4 ITP plasmas generated 1-4% AAE of LT/HETE metabolites. Equal numbers of ITP plasmas produced LT>HETE and LT<HETE; but there was no correlation with the serum anti-glycoprotein (GP) reactions which were specific for GP IIb/IIIa complex alone, for GP Ib+IIb/IIIa, or separate GPs Ib+IIb+IIIa. Monocytes coated with IgG from ITP patients appear to act as effector cells in interactions with platelets. These reactions are a basis for assessing disease

mechanisms and in vitro evaluation of potential therapeutic agents.

Planned work:

a. To complete identification of arachidonate metabolites generated by antibody-mediated monocyte-platelet interactions (AAMPI).
b. To relate antibodies against specific platelet GP epitopes to degree and type of monocyte metabolic response.
c. To define relationships between monocyte responses and source of platelet antigen, whether purified GPs, isolated membranes, or intact cells.
d. To assess pharmacologic agents such as adrenocorticosteroids, nonsteroidal anti-inflammatory drugs and other agents that modify arachidonate metabolism.

2. Serology of ITP

Diagnosis of ITP is based on excluding other possible causes of thrombocytopenia, because there is no laboratory test for the disease. The low concentration and/or affinity of ITP antibodies, the few specific antibodies per platelet (200-500 molecules) leading to in vivo destruction, and the relatively large amounts of nonspecific IgG retained by platelets (500-5000 molecules), have frustrated attempts to develope a serologic test for ITP. The most recently reported tests utilizing monoclonal antibodies to identify platelet glycoproteins (GPs) that react with ITP antibodies are positive in about 40% of chronic ITP patients. We have improved these tests by increasing sensitivity, eliminating the use of monoclonal antibodies, and greatly simplifying and shortening the procedure. The sensitivity and specificity of reactions provides a means of investigating the structural nature of epitopes involved in ITP as well as a basis for more exact serologic diagnosis.

Findings:

Polystyrene beads were coated directly with affinity-purified platelet GPs Ib, IIb, IIIa or the IIb/IIIa heterodimer which account for the major plasma membrane proteins reactive with auto- or alloantibodies. Coated beads were incubated with patient's serum, washed, and developed with ^{125}I-antiglobulin. Of 47 ITP patients, 30 (64%) gave positive reactions with one or more GP. This is the most sensitive test for ITP and appears to detect all plasma antibodies in that >30% of ITP antibodies appear to be bound by platelets with none free. Positive reactions with a single GP were: Ib-18%; IIb-4%; IIIa-2%; IIb/IIIa-6%. Positive reactions with multiple GPs were: Ib+IIb-2%; Ib+IIIa-13%; Ib+IIb/IIIa-4%; Ib+IIb+IIIa-17%. Total reactions for each GP were: Ib-54%; IIb-23%; IIIa-32%; and IIb/IIIa-10% (not including sera positive for IIb or IIIa). Multiple reactions were in all cases due to separate antibodies rather than to a single Ab reactive with the same epitope on different GPs in that adsorption of each serum with a GP in solid-phase removed Ab for only the specific GP used for adsorption.

Planned work:

a. Characterization of the epitope specificities of anti-GPIIb/IIIa and anti-GPIb antibodies by adsorption of sera with peptides generated from known sequence data and enzymatic cleavage sites for these proteins representative of molecular domains having surface, transmembranous, and cytoplasmic orientations.
b. Correlation of clinical data with antibody specificity to evaluate pathogenetic significance.
c. Continued development of new methods of antibody detection including analyses of cell surface IgG on ITP platelets (direct tests) and on normal

platelets preincubated with ITP sera (indirect tests).

3. Naturally Occurring Autoantibodies to Internal Cellular Proteins

Findings:

Last year we reported an intracellular 95 kD platelet protein that reacted with immunoglobulins in over 90% of normal human sera (IgG 85%, IgA 50%, and IgM 30%). These reactions frequently had been interpreted as being specific for disease states since they often mimcked reactions with platelet membrane glycoproteins on Western blots (WBs). We have now identified the 95 kD protein as a fragment of cytosolic/cytoskeletal vinculin and have further identified another cytoskeletal protein, talin (235 kD), as a reactant with immunoglobulins in most normal sera. Based on amino acid sequencing of a tryptic fragment, reactions with monoclonal Ab, pI determination, and electrophoretic profile of its proteolytic fragments, we identified the 95 kD protein as the N-terminal fragment of vinculin (intact MW 117 kD). Vinculin is a cytosolic/cytoskeletal protein found in many cells including smooth, cardiac, and skeletal muscle, platelets, and epithelia. In platelets, calpain proteases appear to be responsible for cleavage of the protein which can occur within minutes of blood collection if EDTA or specific protease inhibitors are not present.

Sera containing high titers of vinculin-reactive Abs may have low or high titers of talin-reactive Abs and vice versa. Identification of these antibody specificities helps interpretation of WBs used clinically to detect pathogenic anti-platelet Abs in sera of patients with immune-mediated destructive thrombocytopenias. The range of vinculin- and talin-Ab titers in patients with immune thrombocytopenias does not differ significantly from that in normals and there is no correlation between titer and severity of TP. We have no evidence that these antibodies play a pathogenic role. They could be secondary markers of platelet injury, but, since vinculin and talin are major components of other cells, antibodies may not be due to sensitization from platelet vinculin and talin per se. Furthermore, we have not ruled out the possibility that they crossreact with some common foreign antigen that, for example, could be bacterial or viral in origin. Since it is difficult to elicit anti-human vinculin and -talin in animals, availability of specific human antibodies will help elucidate the function of these proteins.

Planned work:

a. Purify Abs from high-titered sera by affinity chromatography and use in morphologic studies assessing role of vinculin and talin in shape change and secretion associated with platelet activation.

4. A New Autoimmune Platelet Disorder

Findings:

We studied a patient who presented with chronic gum bleeding and found an high-affinity polyclonal autoantibody to platelet GPIIb/IIIa in her plasma that completely inhibited platelet aggregation. We have found that the Ab inhibits fibrinogen binding to platelets. Her autologous ^{111}Indium platelet survival is normal despite the presence of 20-50 x 10^3 Abs bound per platelet, suggesting there may be a problem with reticuloendothelial clearance. Her underlying disorder appears to be sarcidosis.

Planned work:

a. Investigate status of the patient's reticuloendothelial system by doing a survival of Ab-sensitized RBCs.

b. Attempt to immortalize her Ab (one or more clones) using peripheral mononuclear cells obtained by apheresis and elutriation.

c. Potentially develop this antibody for pharmacologic use as a potent "anti-platelet" agent.

5. Pathophysiology of Posttransfusion Purpura (PTP)

Findings:

PTP is a disease almost exclusively in women caused by alloantibodies against transfused platelet antigens. We earlier reported the finding of nonsedimentable microparticulate platelet-derived antigen (Pl^{A1} antigen) in stored blood and plasma that could be adsorbed in vitro by Pl^{A1}-negative platelets in the presence or absence of anti-Pl^{A1} antibody. This finding indicated that adsorption of antigen-antibody complexes by platelets could occur in patients with PTP. We have now verified that plasmas withdrawn from 6 of 6 PTP patients during periods of thrombocytopenia do indeed contain microparticulate Pl^{A1} antigen from transfused platelets in quantities sufficient to coat autologous antigen-free platelets as antigen-antibody complexes to account for the clinically observed periods of thrombocytopenia. Immunoprecipitation employing MoAbs was used to identify the protein components released from platelets in the form of nonsedimentable microparticles. GPIb, GPIIb/IIIa, GMP-140, and fibrinogen, and IgG are all detectable in this material. Saturation experiments indicate that Pl^{A1}-negative platelets adsorb approx. 50% of the quantity of Ag normally present on Pl^{A1}-positive plts whereas red cells adsorb none. These findings not only explain a disease that has been an enigma because alloantibodies destroyed autologous platelets, but also for the first time demonstrates that membranous components can exchange between cells in vivo to alter antigenicity and function.

Planned work:

a. Development of an animal model for use in studying the *in vivo* mechanism of platelet destruction in PTP.

b. Application of immunogold and biotin-streptavidin labels in electron microscopy to characterize morphology of nonsedimentable transferrable platelet material.

c. Study the possible functional roles of platelet microparticles in primary and secondary hemostasis.

d. Measure amounts of platelet micropaticulate material in plasma from patients with destructive thrombocytopenias such as ITP.

References:

1. Reid DM, Jones CE, Vostal JG, Shulman NR. Western blot identification of platelet proteins that bind normal serum immunoglobulins. Characteristics of a 95 kD reactive protein, Blood 1990;75:2194-2203.

2. Shulman NR. Post-transfusion purpura: clinical features and the mechanism of platelet destruction, AABB Seminar Series, in press.

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Objectives:

To study reactions and interactions of coagulation factors *in vitro* and *in vivo*, to define further the nature of the blood coagulation mechanism, and to study the physiology and biochemistry of platelet responses to agonists causing exocytosis and aggregation. These studies are aimed at determining factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis and at developing better forms of therapy for these diseases.

Methods Employed: Methods of protein purification and characterization, techniques of enzymology, proteolytic enzymes and their inhibitors, kinetic analysis of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactive substrates, particularly arachidonate metabolism and tyrosine phosphorylation. Tissue culture techniques, pharmacologic and physiologic studies of cellular secretion, and platelet aggregation. SDS-PAGE molecular weights, amino acid composition of purified proteins, immunologic RIA, fluorescent, Western Blot, EIA and other quantitative and qualitative methods of identifying specific epitopes with antibodies, HPLC, affinity columns, immunoprecipitation, isoelectric focusing, tissue culture, lymphocyte transformation, Northern Blots, DNA labeling.

1. Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins.

Findings:

Depletion of intracellular calcium stores appears to increase plasma membrane permeability for calcium by an as yet obscure mechanism. We found that the Ca^{++} ionophore, A23187, and thrombin elevate cytosolic calcium ($[Ca^{++}]_i$) equally and cause tyrosine phosphorylation of a 130-kD protein and to a lesser extent 80- and 60-kD proteins. Chelation of ($[Ca^{++}]_i$) by 1,2-bis(2-aminophenoxyethane)-N,N,N',N'-tetraacetic acid/acetomethoxy ester decreased thrombin-induced tyrosine phosphorylation responses. These results suggested that ($[Ca^{++}]_i$) elevation promotes tyrosine phosphorylation. Tyrosine phosphorylation persisted in the presence or absence of extracellular calcium after thrombin stimulation, but subsided rapidly after A23187 addition if extracellular calcium was present. When Ca^{++}/ATPase activity, which is apparently required to maintain calcium stores, is inhibited by low temperature, tyrosine phosphorylation of the 130-kD protein occurs. Rewarming platelets reverses tyrosine phosphorylation only if extracellular calcium is present. Thapsigargin, a calcium ATPase inhibitor, also induces tyrosine phosphorylation of the 130-kD protein and prevents dephosphorylation of this protein when added prior to rewarming. These observations suggest that homeostatic levels of calcium in storage compartments favor tyrosine dephosphorylation of specific proteins. Thus the levels of ($[Ca^{++}]_i$) and stored calcium appear to control tyrosine phosphorylation antagonistically. Tyrosine phosphorylation appears to play a role in regulating calcium channel function; and the 130 kD protein, when phosphorylated, may facilitate opening the plasma membrane channel.

Planned work:

a. Isolate the 130 kDa protein which is the major protein phosphorylated during platelet activation and dephosphorylated during regeneration of Ca^{++} stores. This work is currently underway.

b. Clone the protein.

c. Determine its specificity as a substrate with respect to protein kinases, possible function as a Ca^{++} channel, responses to specific kinase and phosphatase

2. Biochemical evidence of activation of monocytes by thrombin

Findings:

Monocytes isolated by elutriation were incubated for 18 hours at 25°C with 1μCi/ml of ^{3}H-arachidonic acid, washed, incubated in IMDM medium containing human serum albumin and stimulated for 2 hours at 37°C with one unit of thrombin per ml. Arachidonic metabolites in the supernatant fluid of stimulated monocytes were analyzed by HPLC utilizing a C-18 3μm silica bead column; prostaglandins were eluted with phosphoric acid linear and step gradients and leukotrienes and HETES with a MeOH step gradient. Arachidonate metabolites were identified by comparison with appropriate standards and quantified with an in-line liquid scintillation counter. These resoponses did not occur in the presence of hirudin which binds thrombin in proximity to its catalytic site and inhibits its clotting and chemotactic activities. The arachidonate metabolites observed can cause capillary leakage and modulation of Fc receptors.; and in murine sysrems, are associated with interleukin (cytokine) production. This work has defined an interconnection between blood coagulation and inflammation that will contribute to further understanding of atherosclerosis and other forms of vascular occlusion or leakage.

Planned work:

a. To identify the specific leukotrienes and HETEs formed by human monocytes stimulated with thrombin.

b. To determine whether thrombin-stimulated human monocytes form cytokines or growth factors which can be cytotoxic, proliferative, or stimulatory.

c. To determine whether eicosanoids produced by monocytes in the presence of thrombin activate platelets or leukocytes.

References:

1. Vostal JG, Jackson WL, Shulman NR. Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins, J Biol Chem 1991;266:in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 51,000- 33 CHB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Study of Immunology of Blood Cell Deficiencies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	Diane M. Reid	Staff Fellow	CHB:NIDDK
	Charles E. Jones	Chemist	CHB:NIDDK
	R.-Y. He	Visiting Fellow	CHB:NIDDK
	William Jackson	Research Fellow	CHB:NIDDK

COOPERATING UNITS *(if any)*

Charles Carter (CC-DTM); J. Bussell, N.Y. Hospital; J. Blanchette-Mackie (LCDBM-NIDDK)

LAB/BRANCH

Clinical Hematology Branch

SECTION

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mechanisms of immune platelet destruction in ITP are obscure. Macrophages may participate in platelet destruction since inhibition of splenic function is therapeutic. We developed a method to directly test macrophage participation in immune platelet reactions. Monocytes armed with antiplatelet ITP- or allo-antibodies against platelet membrane glycopoteins (GPs) Ib, IIb, and IIIa, when exposed to platelets, secreted arachidonic acid metabolites [leukotrienes, and hydroxyeicosanoic acids (HETEs)], but did not form cytokines. Monocyte responses can be used to further assess disease mechanisms and to evaluate therapeutic agents in ITP.

There has been no reliable serologic test for antiplatelet antibodies in ITP. By coating polystyrene beads with purified platelet membrane GPs that react with ITP antibodies followed by ^{125}I-antiglobulin, we found positive reactions in 64% of ITP sera; an additional 30% are platelet-bound. Antibodies were often multiple, directed against GPIb, IIb, IIIa, and IIb/IIIa heterodimer. This provides not only effective serologic diagnosis, but also insight into structural aspects of ITP epitopes.

We found that Igs in over 90% of normal sera react with a 95 kD platelet protein on Western blot, which by amino acid sequencing was an N-terminal fragment of vinculin; and that Igs in 60% react with talin. These antibodies have complicated diagnosis of immune disorders but are not disease-associated; they can be used to study functions and structure of the major cytoskeletal proteins.

We defined a new autoimmune platelet disorder caused by an autoantibody to platelet GPIIb/IIIa that inhibits platelet aggregation by preventing fibrinogen binding. This antibody potentially can be developed for pharmacologic use as a potent "anti-platelet" agent.

Posttransfusion purpura, a disease almost exclusively of women, which we described initially, was further elucidated by finding that microparticulate platelet antigen (Ag) from transfused platelets that coats autologous Ag-free platelets as an Ag-Ab complex to cause thrombocytopenia.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 51,001-33 CHB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Raphael Shulman	Chief	CHB:NIDDK
Others:	Jaroslav Vostal	Research Fellow	CHB:NIDDK
	William Jackson	Research Fellow	CHB:NIDDK

COOPERATING UNITS *(if any)*

Charles Carter (CC-DTM)

LAB/BRANCH

Clinical Hematology Branch

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Platelets prevent hemorrhage by a secretory process which is stimulated by an intracellular chain of biochemical events similar to that in all other secretory cells. Although tyrosine kinases (TKs), which control placement of phosphates on tyrosine residues of protein, are associated with major biological effects such as cell growth, receptor activity, and oncogene mutation, biochemical mechanisms underlying these events are only partially known. We found that high levels of cytoplasmic Ca^{++} promotes tyrosine phosphorylation, particularly of a 130 kD cytoplasmic platelet protein, whereas a homeostatic level of calcium in storage compartments activates tyrosine dephosphorylation of the same protein. Finding that levels of cytoplasmic and stored Ca^{++} affect tyrosine phosphorylation by controlling phosphatase activity is the first recognition of a specific physiologic role for a tyrosine phosphatase (TP). Because reciprocal interaction between a TK and a TP appears to control Ca^{++} flux and phosphorylation of the 130 kDa protein, this protein may be involved in Ca^{++} channel function. Other significant cellular events such as cell growth, cell-to-cell interaction, contact inhibiton, and malignant transformation may depend on relationships we have defined between TKs, TPs, Ca^{++} distribution, Ca^{++} flux, and secretion.

Linkage between the coagulation process and inflammation is suggested by vascular damage and intravascular clotting that occurs in septic shock and by infiltration of inflammatory cells in areas of vascular damage due to atherosclerosis. Activation of platelets by clotting factors is well characterized but whether clotting factors can activate monocytes and leukocytes is not known. We found that reaction between thrombin and a thrombin receptor on cultured monocytes causes generation of prostaglandins, leukotrienes, and HETEs, which are products of monocyte activation. This response is mediated by the thrombin site that binds hirudin. These findings indicate that release of monocyte interleukins and tissue necrosis factor may be the link between coagulation, inflammation, and the vascular damage evident in septic shock and atherosclerosis.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

ANNUAL REPORT OF THE GENETICS AND BIOCHEMISTRY BRANCH

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Biochemical Genetics Section

Dr. Proia and his colleagues have continued their studies of the lysosomal enzyme β-hexosaminidase (a deficiency of which is responsible for Tay-Sachs disease). This enzyme is made up of two subunits α and β. Over the last few years they have cloned the cDNAs and genes for both chains of the human β-hexosaminidase. In the last year they have elucidated the complete organization of the murine HEXB gene and the majority of the HEXA gene. This has allowed the construction of gene targeting vectors that are being used to disrupt the HEX genes in mouse embryonic stem cells. The ultimate purpose is to produce mouse models for the different forms of Tay-Sachs.

In addition, Dr. Proia and his colleagues have examined six unrelated Moroccan Jewish carriers of Tay-Sachs and have identified three HEXA gene mutations in this population: (1) a Leu^{181}->Stop mutation in one carrier, (2) an Arg^{170}->Glu change in two of the carriers and (3) an in-frame deletion of a phenylalanine codon at position 304 (ΔF) in three of the carriers. The Leu^{181}->Stop and ΔF are new HEXA mutations. The Arg^{170}->Glu change had been found previously in a Japanese patient and occurs at a CpG mutational hot spot.

Finally, Dr. Proia and members of his group have investigated the mechanisms responsible for the retention of proteins in the endoplasmic reticulum. They have identified the retention signals for a family of carboxylesterases that are variants of the yeast signal (HDEL), a signal not previously known to be utilized by mammalian cells.

Molecular Genetics Section

Dr. Ackerman and collaborators have continued their work on the mode of action of the *Aspergillus* toxin α-sarcin and other substances that inhibit protein synthesis. They had previously demonstrated that several toxins all inactivate protein synthesis in cells by attacking a highly conserved sequence in 28S ribosomal RNA. They had also shown that modified oligonucleotides and ribozymes complementary to the same region of 28S RNA also inhibited protein synthesis. They have now demonstrated that certain nucleases are surprisingly efficient in abolishing protein synthesis and that one of these nucleases selectively hydrolyzes tRNA when injected into *Xenopus* oocytes.

On a different project Dr. Ackerman and his colleagues have extended their studies on DNA repair. They have demonstrated pyrimidine dimer repair in *Xenopus* oocytes by injecting UV-irradiated DNAs; in a cell-free system they have detected dimer repair by using an extract derived from oocytes. They are studying the role of different DNA polymerases in their repair extract.

Dr. Hsieh and her colleagues are examining the effects of mismatched bases on DNA branch migration. When heteroduplex DNA is formed in the course of the recombination or repair of DNA the heteroduplex regions can undergo branch migration. Depending on the direction of this branch migration, the length of the heteroduplex region will increase or decrease. Longer heteroduplex regions will result in an increase in the amount of genetic information that is exchanged between homologues. Using three-stranded substrates, Dr. Hsieh has observed that protein-free branch migration is impeded by as few as two mispaired bases (mismatches or deletions). Furthermore, they find that the *E.coli* recA protein, a protein that is responsible for creating such heteroduplexes in *E.coli*, does not appear to significantly promote branch migration through such mispaired bases. On the basis of these results, they suggest that recombination proteins such as recA promote the accommodation of mismatched bases and short deletions in heteroduplexes by nucleating pairing of homologous DNAs in the regions flanking these subsequent mispairings.

Dr. Camerini-Otero and his colleagues continue their studies aimed at dissecting the biochemical steps involved in genetic recombination. Over the last few years they have focused on identifying the proteins responsible for recombination and how they work. They have chosen to concentrate their efforts on a key early step: strand exchange between homologous parental DNAs. *In vitro*, the product of this exchange is a joint molecule composed of a single-strand DNA joined to the end of a duplex DNA. First they identified several eukaryotic proteins responsible for this reaction. Recently they have established a new paradigm of this homologous pairing: that all recombinases, including the *E.coli* recA protein, can hybridize a single strand of any sequence and an intact duplex. That is, the three strands form a novel DNA triplex (which they have designated R-form DNA) in which the third strand may include both purines *and* pyrimidines.

In the last year, they have carried out three critical tests of the structural model of R-form DNA. First, using chemical footprinting they have demonstrated for the first time hydrogen bonding between the third strand and the identical strand in the Watson-Crick duplex in homologous recombination. Second, using duplexes containing chemically substituted bases they have proven the importance of certain atoms to the thermal stability of R-form DNA. Finally, they have developed a method to selectively cleave duplex genomic DNA based on their ability to form R-form DNA: RecA Assisted Restriction Endonuclease (RARE) cleavage. RARE cleavage was used to excise the cystic fibrosis gene from the human genome with a fragment yield of about 80%.

Finally, Drs. Camerini-Otero and Proia and colleagues have examined the role that glycosylation plays in the expression of CD4, a 55kD monomeric membrane-bound glycoprotein expressed on the surface of T Helper cells. This protein is a member of the immunoglobulin supergene family, mediates helper T cell activation and is also the receptor of the human immunodeficiency virus (HIV). The posttranslational attachment of oligosaccharide is required for proper folding and transport of many secreted and integral membrane proteins. Using a series of mutant cDNAs for CD4 they have demonstrated that: (1) both potential glycosylation sites are utilized; (2) glycosylation at either site is necessary and sufficient for proper protein folding and transport to the cell surface; and (3) unglycosylated CD4 is found in cells at levels comparable to wild type CD4 but is not properly folded and is not transported to the cell surface (that is, is retained intracellularly).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52008-12 GBB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Expression and Human Genetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.D. Camerini-Otero	Chief	GBB, NIDDK
Others:	P. Hsieh	Expert	GBB, NIDDK
	C.S. Camerini-Otero	Medical Officer	MDB, NIDDK
	R. Gardner	Clinical Associate	GBB, NIDDK
	E. Angov	IRTA Fellow	GBB, NIDDK
	M. Kim	Visiting Fellow	GBB, NIDDK
	L. Ferrin	Research Associate	GBB, NIDDK
	S. Pati	IRTA Fellow	GBB, NIDDK
COOPERATING UNITS *(if any)*	R. Kiyama	Visiting Fellow	GBB, NIDDK

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
Molecular Genetics Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.25	7.75	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to dissect the biochemical steps involved in genetic recombination we have chosen to focus on a key early step: strand exchange between homologous parental DNAs. *In vitro*, the product of this strand exchange reaction is a joint molecule composed of a single-stranded DNA joined to one end of a linear duplex DNA. We have established a new paradigm for this homologous pairing, in essence, that recombinases such as the *E. coli* recA protein can hybridize a single strand of any sequence to an intact duplex. That is, the three strands form a novel DNA triplex (R-form DNA) in which the third strand may include both purines *and* pyrimidines.

We have carried out three critical tests of the structural model of R-form DNA. First, using chemical footprinting we have demonstrated for the first time the hydrogen bonding between the third strand and the identical strand in the Watson-Crick duplex in homologous recombination. Second, using duplexes containing chemically substituted bases we have proven the importance of certain atoms to the thermal stability of R-form DNA. Finally, we have developed a method to selectively cleave duplex genomic DNA based on our ability to form R-form DNA: RecA Assisted Restriction Endonuclease (RARE) cleavage.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-930

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52011-06 GBB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toxins and DNA Repair in *Xenopus* Oocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric Ackerman	Senior Staff Fellow	GBB, NIDDK
Others:	Shailendra K. Saxena	Visiting Associate	GBB, NIDDK
	Jitendra K. Saxena	Visiting Associate	GBB, NIDDK
	Timothy M. Jenkins	Visiting Fellow	GBB, NIDDK
	Joshua D. Levin	IRTA	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.50	3.25	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. We previously demonstrated that the toxins α-sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA. We have now shown that microinjection of modified deoxyoligonucleotides, ribonucleotides and ribozymes complementary to the same region of 28S RNA also abolish protein synthesis. We have also demonstrated that certain nucleases are surprisingly effective at abolishing protein synthesis and that one of these nucleases selectively hydrolyzes tRNA, but not ribosomal or mRNAs, when injected into *Xenopus* oocytes.

II. During early development *Xenopus* replicates its DNA nearly as fast as *E. coli* in log phase; perhaps indicating that oocytes may be an excellent source of DNA repair activity. We demonstrated pyrimidine dimer repair by microinjecting UV-irradiated plasmid DNA into oocytes or adding damaged DNA to an extract derived from oocytes. We have also studied DNA repair for alkylated and chemically modified DNA. We are also investigating DNA replication with our repair extract.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52012-07 GBB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Structure-Function Relationships of Lysosomal Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard L. Proia	Research Biologist	GBB, NIDDK
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	Sukumar Medda	Senior Staff Fellow	GBB, NIDDK
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	Olivia Johnson	Summer IRTA	GBB, NIDDK

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Ruth Navon, Saphir Medical Center, Kfar-Sava, Israel

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Biochemical Genetics Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	4.0	0.25

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. The Moroccan Jewish population has an elevated incidence of Tay-Sachs disease. We have examined 6 unrelated Moroccan carriers of the disease and have identified three HEXA gene mutations: (1) a Leu^{181}->Stop mutation in one carrier, (2) an Arg^{170}->Glu change in two of the carriers and (3) an in-frame deletion of a phenylalanine codon at position 304 (ΔF) in three of the carriers.

II. The complete organization of the murine HEXB gene and the majority of the HEXA gene has been established. This has allowed the construction of gene targeting vectors for the purpose of "knocking out" the HEX genes in mouse embryonic stem cells and for ultimately producing mouse models of the G_{M2} gangliosidoses.

III. Mechanisms by which normal and abnormal proteins are retained in the endoplasmic reticulum (ER) have been determined. The protein signals that specify whether members of a family of carboxylesterases are secreted or retained in the ER have been identified. These retention signals are variants of the yeast signal (HDEL) previously thought not to function in mammalian cells.

We have also found that the unglycosylated α-subunit of β-hexosaminidase undergoes an altered folding pathway resulting in the formation of an insoluble, inappropriately disulfide-bonded aggregate that is retained in the ER. The inappropriately folded protein is bound to BiP/GRP 70 which acts as a molecular scaffold in ER.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52014-04 GBB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

CD4 Receptor Structure/Function Project

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Daniel Camerini-Otero	Chief	GBB, NIDDK
Others:	Richard L. Proia	Research Biologist	GBB, NIDDK
	Cynthia Tifft	Clinical Associate	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The posttranslational attachment of oligosaccharides is required for proper folding and transport of many secreted and integral membrane glycoproteins. Human CD4, the 55kD protein present on a subset of T lymphocytes and the receptor for the human immunodeficiency virus (HIV), contains two potential asparagine-linked glycosylation sites within its extracellular domain. Previous work has suggested that glycosylation, is required for cell surface expression of CD4 and that unglycosylated CD4 is unstable and rapidly degraded. Other studies have shown that unglycosylated CD4 can function as a cell surface receptor for HIV.

To clarify and further define the role of glycosylation we generated a series of mutant cDNAs in which one, the other, or both potential glycosylation recognition sites were eliminated. Using *in vitro* transcription and translation we confirmed that both potential glycosylation sites of CD4 are utilized. We transiently expressed the mutant cDNAs in human cells and precipitated the resulting proteins using monoclonal and polyclonal antibodies to CD4. Using this approach we demonstrated that glycosylation at either site was necessary and sufficient for proper protein folding and transport to the cell surface. We have found that unglycosylated CD4 is synthesized in the cell at levels comparable to wild type CD4 and can be precipitated only by the polyclonal antiserum, suggesting that unglycosylated CD4 is improperly folded and retained intracellularly, probably in the endoplasmic reticulum (ER).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52015-02 GBB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Molecular Studies of Protein-DNA Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Peggy Hsieh	Expert	GBB, NIDDK
Others:	Susan J. Landsman	GS-7 Technician	GBB, NIDDK
	Tammy Tobin	IRTA	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.85	2.75	0.1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are examining the effects of mispaired bases on DNA branch migration. During homologous recombination, Holliday structures containing regions of heteroduplex DNA are formed. These heteroduplex regions can undergo branch migration which in one direction, can increase the amount of genetic information that is exchanged between homologous chromosomes. Alternatively, net movement in the reverse direction will reduce the length of heteroduplex DNA. We are examining the role of *E.coli* recA protein in promoting branch migration through mispaired bases. In order to do so, we have initiated studies of nonenzymatic branch migration through mispaired bases. Using oligonucleotides to form three-stranded substrates, we observe that nonenzymatic branch migration does not proceed to any significant extent through as few as two mispaired bases (mismatches or deletions). An examination of branch migration in the presence of RecA nucleoprotein filaments formed in the presence of ATP reveals that RecA has only a very modest effect in promoting branch migration through mispaired bases. On the basis of these results, we suggest that recombinase proteins like RecA can accommodate mispaired bases in heteroduplex DNA by nucleating pairing of homologous DNAs in regions flanking mispaired bases. We are currently studying branch migration using four-stranded substrates.

We are also initiating studies on the regulation of expression of the human ligase I gene. We have obtained cDNA probes corresponding to various regions of the ligase gene using Polymerase Chain Reaction (PCR) and are examining by Northern blot analysis whether the ligase gene is activated in response to UV damage in HeLa cells.

Annual Report of The Digestive Diseases Branch
National Institute of Diabetes, and Digestive and Kidney Diseases

SUMMARY OF BRANCH ACTIVITIES

All four sections of the Digestive Diseases Branch are engaged in investigations of basic biologic processes (e.g., hormone action, membrane transport, cellular and humoral immunology) and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver, pancreas and gastrointestinal tract. All four sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, hepatitis and fulminent hepatic failure. Below the important results found in the various sections are briefly summarized with more detailed descriptions in the individual section reports.

SECTION ON GASTROENTEROLOGY

Recent studies suggest that cell surface receptors for CCK as well as a number of gastrointestinal hormones are internalized. Studies show there are two classes of CCK receptors, a high affinity and a low affinity class. The relationship of these to the biphasic enzyme release dose-response curve is unknown. To explore the possibility that there may be recycling of CCK receptors after internalization, and to possibly gain insight into the cellular function of the different CCK receptor classes, the effects of monensin were investigated on CCK binding, because this agent has been shown to inhibit recycling of cell surface receptors in a number of systems. Preincubation with monesin inhibited binding of ^{125}I-BH-CCK-8 with a maximal effect at 90 min. Monesin caused an 84% decrease in the number of high affinity CCK receptors with no change in the number of low affinity receptors. First inhibiting pancreatic acinar with monesin did not alter the ability of CCK to stimulate enzyme secretion. These results were interpreted to indicate that high affinity CCK receptors recycle, whereas the low affinity do not, and that low affinity receptors mediate CCK-stimulated enzyme secretion. By comparing the binding inhibition curves and enzyme secretion dose-response curves it was concluded that pancreatic acini may possess three classes of CCK receptors, a high affinity, low affinity and very low affinity class. The function of the high affinity receptors is not known, the low affinity moderates the upstart of the biphasic amylase release dose-response curve and the very low affinity the downstroke.

Studies by us and others have shown that occupation of a given receptor by a gastrointestinal receptor agonist may affect the ability of another agonist at a different receptor to alter cell formation (heterologous densensitization). During the last year we demonstrate that muscarinic cholinergic agonists can cause down-regulation to a number of receptors in pancreatic acinar cells including receptors for CCK, muscarinic cholinergic agents, and bombesin, but not to substance P receptors.

I. Studies relating to the identification and characterization of receptors for gastrointestinal peptides.

I.A. Development of specific receptor antagonists for GI hormones.

I.A.1. Development of bombesin (Bn) receptor antagonists.

Bombesin (Bn), neuromedin B (NMB), gastrin-releasing peptide (GRP) and related peptides have numerous pharmacologic actions in the GI tract and CNS such as regulating temperature control, causing tumor growth, releasing numerous other GI peptides and affecting motility. Which of these functions are physiological are unknown because of the lack of antagonists.

In previous studies we have discovered, in collaboration with Dr. D.H. Coy (Tulane University, School of Medicine, Peptide Research Lab) and Biomeasure Corporation, Hopkinton, MA., four different classes of bombesin receptor antagonists. During the last year it has been possible to develop even more potent Bn receptor antagonists which have antagonist activity in all species examined.

Current studies demonstrate that with both Bn and with the structurally-related peptide litorin that the COOH-terminal amino acid is important for initiating a biological response but not essential for determining receptor affinity. Structure-function studies of des Met^{14} Bn analogues demonstrated that various ester, amide and alkylamide analogues were potent antagonists of the action of Bn in a number of cell systems and in vivo. Our results suggested that the position 13-CO group of Bn, at least in des Met^{14} analogues, might be involved in binding to the Bn/GRP receptor via hydrogen binding. This conclusion was supported by the observation that the affinity of analogues could be enhanced by adding electron-releasing substitutions at the des Met^{14} terminus and decreased with substitutions such as a free carboxyl group perhaps because the CO electrons were being distributed over two O groups.

Additional structure-function studies of various des Met^{14} Bn analogues demonstrated that the insertion of certain D-amino acids in the 6 position of Bn from the COOH-terminus, such as [D-Phe^6], resulted in truncated Bn(6-13) analogues which had equal or greater affinity than Bn(1-13) analogues, for the Bn receptor. Some of the Bn analogues such as [D-Phe^6]Bn(6-13)methyl ester were shown to also be potent antagonists in vivo and because they offer fewer proteolytic degradation sites and are relatively short peptides, they should be useful for in vivo studies.

Previous studies demonstrated that pseudopeptide analogues of either the COOH-terminus of Bn or the structurally-related peptides, gastrin-releasing peptide (GRP) or litorin were another class of potent Bn receptor antagonists in a number of cell systems. Recent studies suggested some of these analogues might have partial agonist activity in some species thus limiting their general usefulness. In detailed structure-function studies using rat pancreatic acini which possess a Bn receptor which has much less stringent peptide requirements for Bn receptor activation than that on 3T3 cells or guinea pig pancreatic acini, the ability of various pseudopeptide analogues of Bn-related peptides (i.e. with the -CONH- bond in position 13 reduced to $-CH_2NH-$) to initiate a biologic response was investigated. It was found that the insertion of either a D-Phe^{14} or p-chloro Phe^{14} resulted in potent antagonists in all species. Therefore, peptides such as [D-Phe^{6,14},ψ 13-14]Bn(6-14) or [D-Phe^6,Cpa^{14}]Bn(6-14) were discovered to be potent antagonists in all species examined and thus might also be useful for in vivo studies.

It is extremely difficult to precisely evaluate by physiochemical measurements the configurational structure of short peptides with flexible polypeptide chains, which for example, in the case of bombesin in high resolution NMR or CD studies, show little ability to adopt preferred conformation in solution. A partial solution to this problem with peptides such as Bn would be to develop conformationally restricted analogues which are less flexible while retaining ability to interact with their receptors. During the last year in an attempt to do this with bombesin-related peptides, a series of shortened bombesin receptor agonists and antagonists were cyclized at different points by utilizing disulfide bonds or by head to tail cyclization. A number of the cyclized analogues retained agonist activity with one analogue having a relatively high affinity of 67 nM. One analogue retained antagonist activity (K_i - 1.2 μM). These cyclized analogues peptides have a marked increase in conformational restraint and thus should be useful substrates for NMR and molecular modeling studies aimed at comparing differences between agonist and antagonist structure. These should service as useful starting points to design specific agonists/antagonists based on knowing the biologically active configuration.

I.A.2. Characterization and/or development of receptor antagonists for other gastrointestinal peptides.

Secretin has numerous actions in both the CNS and GI tract such as affecting pancreatic growth, stimulation of pepsinogen and pancreatic bicarbonate release, and having potent effects in the kidney, heart and CNS. Which of these are physiological or pharmacological is unknown because of the lack of available antagonists. Existing secretin receptor antagonists are of low potency and specificity. In collaboration with Dr. D.H. Coy (Tulane University) a new class of secretin receptor antagonists has been discovered. The ψ4,5, ψ2,3 and ψ8,9 analogues of secretin had no agonist activity; however, each inhibited 1 nM secretin-stimulated cAMP in guinea pig pancreas. The reduced peptide bond analogue [ψ4,5] secretin was the most potent antagonist (K_i=4 μM) and was selective for peptides interacting with the secretin or VIP receptor. These studies suggest that in the future it is likely that pseudopeptide analogues of secretin might be found which will be more potent and have even higher selectivity for secretin receptor and therefore will be of value in evaluating the physiological function of secretin in various processes.

I.B. Identification and characterization of receptors for gastrointestinal peptides.

Recent studies by our laboratory and in collaboration with others have provided evidence that similar to receptors mediating the action of a number of other gastrointestinal peptides, more than one subtype of Bn receptor exists. On both rat pancreatic acini and guinea pig gastric smooth muscle cells, a Bn receptor with high affinity for GRP, bombesin and neuromedin C and low affinity for neuromedin B (GRP-preferring) was identified by using binding studies or functional assays with various antagonists. On rat esophageal muscularis mucosa and isolated gastric smooth muscle cells from the guinea pig a Bn-receptor with greater than a 100-fold higher affinity for NMB than GRP or neuromedin C was identified by autoradiographic studies, binding studies or functional assays using various agonists and antagonists. Recently two different receptors for this family of peptides have been cloned. A GRP-preferring receptor with high affinity for GRP and low affinity for NMB was cloned from 3T3 cells in 1989 by two different groups. In 1990, Jim Battey in NIMH made a cDNA library from a rat esophagus because our group had described the presence of a NMB-preferring bombesin receptor in this tissue. Subsequently, he cloned a NMB-preferring bombesin receptor from this library after using low stringency hybridization screening to identify a clone. In collaboration with Jim Battey the uniqueness of this receptor was partially established using selective Bn receptor antagonists and radioligands that we have previously described.

I.C. Identification and characterization of receptors for gastrointestinal hormones using various radioligands.

Recent collaborative studies with Dr. Terry W. Moody, Biochemistry Department, Georgetown University and Dr. Tim Moran, Department of Psychiatry, Johns Hopkins University, have provided evidence that at least two subtypes of Bn receptors also are present in the central nervous system. Similar to gastrointestinal tissues, one type of Bn receptor is neuromedin B-preferring and is the most prevalent Bn receptor in the CNS. The other type Bn receptor in the CNS is GRP-preferring with preliminary studies suggesting primarily this type of Bn receptor in the nucleus accumbens and olivary nucleus in the hindbrain. Because both interact with high affinity with Bn it is likely each is important in causing many of the pharmacological effects in the CNS caused by Bn.

We have discovered that the four different classes of Bn receptor antagonists previously described by our group have markedly different affinities for the two Bn receptor subtypes. The antagonists [D-Phe^{6}]Bn(6-13)ethyl ester, [D-Phe^{6},Cpa^{14},ψ13-14]Bn(6-14), Leu^{14},ψ13-14]Bn and [Leu^{14},ψ9,10]Bn have a 10,000-, 1425-, 122- and 40-fold, respectively, higher affinity for Bn receptors that were GRP- or NMC-preferring than Bn receptors that were neuromedin B-preferring. In contrast, the antagonists [Tyr^{4},D-Phe^{12}]Bn, [D-Pro^{4},D-Trp^{7,9,10},- Leu^{11}]SP-4-11 and [D-Arg^{1},D-Trp^{7,9},Leu^{11}]SP had a 4-, 7- and 9-fold respectively, higher affinity for Bn receptors that were neuromedin B-preferring than Bn receptors which were GRP-preferring. Some of these antagonists have been shown to be active in vivo, and thus will allow the subtype of Bn receptor mediating different physiological processes to be differentiated.

II. Studies related to mechanism of the ability of gastrointestinal peptides to alter cellular function.

In previous studies we and others have shown that cholecystokinin (CCK), interacts with specific high affinity receptors on pancreatic acinar cells, activates phospholipase C, stimulates increases in phosphoinositides and mobilizes cellular calcium which results in enzyme secretion. To gain additional insight into the ability of CCK to alter cell function, the kinetics and stoichiometry of CCK and a related peptide, CCK-JMV-180 [Boc-Nle^{28,31},CCK(27-32)-2 phenylethyl ester, an analogue of CCK with partial agonist activity] to stimulate the accumulation of inositol phosphates, mobilize cellular calcium and stimulate enzyme secretion were compared. CCK-8 caused a rapid increase in [^{3}H]IP$_{2}$ and [^{3}H]IP$_{3}$ (2-fold over basal at 10 sec), and HPLC analysis demonstrated at 10 sec, 100% of the [^{3}H]IP$_{3}$ was IP$_{3}$(1,4,5). CCK-JMV-180 caused no increase in [^{3}H]IP$_{3}$ at 10 sec; however, by a sensitive radioreceptor mass assay a 3-fold increase was detected. Each peptide mobilized cellular Ca, although CCK-JMV-180 was only 45% as effective as CCK. Comparison of the abilities of each peptide to stimulate IP$_{3}$(1,4,5) accumulation, to mobilize cellular Ca and stimulate enzyme secretion demonstrated that CCK-related peptides cause maximal enzyme secretion with minimal changes in calcium mobilization and maximal changes in calcium mobilization with minimal changes in IP$_{3}$(1,4,5), suggesting marked amplification.

In the past it has been difficult to investigate interaction with receptors on small localized tissues because of the heterogeneity of cell types in the GI tract. Recently we developed a tissue section method which allowed us to perform receptor characterization studies on small pieces of tissues and investigate precise localization using autoradiography. One of the primary physiological functions of the hormone, CCK, is to influence the contractile state of the sphincter of Oddi (SO). To characterize directly the ability of CCK to interact with specific receptors on the SO, we measured binding of ^{125}I-BH-CCK-8 to tissue sections from the guinea pig SO. Autoradiography localized binding of ^{125}I-BH-CCK-8 over the smooth muscle layer of the sphincter. Binding of ^{125}I-BH-CCK-8 to SO tissue sections was saturable, specific, dependent on time, pH and temperature, and was reversible. Binding of ^{125}I-BH-CCK-8 was inhibited by various CCK receptor agonists with the following potencies: CCK-8 >des(SO_3)CCK-8 >gastrin-17-I and by various CCK receptor antagonists with the following potencies: L-364,718 >proglumide analogue 10 >CBZ-CCK-27-32-NH_2 >Bt_2cGMP. Analysis of binding of ^{125}I-BH-CCK-8 to SO tissue sections revealed two classes of CCK binding sites: a high affinity site (K_d 0.2nM), and a low affinity site (K_d 70nM). Atropine (0.1μM) or tetrodotoxin (1μM) caused a similar rightward shift of the CCK-8-dose-response curve for stimulation of contraction of the SO. Comparison of receptor occupation to CCK-8-induced contraction suggested that the high affinity binding site correlated with the ability of CCK-8 to cause contraction in the presence of atropine or tetrodotoxin. These results suggest the possibility that high affinity CCK binding is to neural elements which mediates atropine- and tetrodotoxin-sensitive SO contraction, whereas low affinity binding is to muscle mediates atropine- and tetrodotoxin-insensitive SO contraction.

Recent studies suggest that the COOH terminal amino acid of Bn or GRP is crucial for initiating a biologic response at the GRP-preferring Bn receptor but not for determining affinity. In collaboration with Dr. David H. Coy, Peptide Research Labs, Tulane University, we examined further the important structural determinants in Bn-related peptides for determining Bn receptor affinity for GRP-preferring Bn receptors in a number of cell systems and the ability to initiate biologic responses by synthesizing 18 des Met^{14} Bn(6-13) analogues. With both guinea pig acini and 3T3 cells, which have GRP receptors with high peptide stringency structural requirements for cell activation, receptor affinity was affected by the chain length of the alkyl moiety (R) added to [D-Phe^6]Bn(6-13)NH_2R, whereas in rat pancreatic acini a cell system with low peptide stringency structural requirements for cell activation receptor affinity was not. The affinity in all 3 cell systems was increased by additions of other electron releasing groups to the COOH-terminal carboxyl group such as [D-Phe^6]Bn(6-13)ethyl or methyl ester or hydrazide. Comparison of the ability of the various analogues to alter biologic activity in guinea pig pancreas and 3T3 cells revealed that 12 were antagonists, 1 a full agonist and 5 were partial agonists. In rat acini 8 were antagonists, 5 full agonists and 5 partial agonists. Potent antagonists in each cell system were the methyl and ethyl ester, hydrazide and ethylamide analogues. In 3T3 cells or guinea pig pancreas, agonist activity of the alkylamide was critically dependent on the chain length, whereas with rat pancreatic Bn receptors any alkylamide longer than the ethylamide had agonist activity. These results further demonstrate that the nature of the substitution on the carboxyl terminal of des Met^{14} Bn analogues is critically important, not only for determining Bn receptor affinity but also for determining the ability to initiate a biologic response. In contrast to previous studies, the present results demonstrate that the presence of the COOH terminal amino acid in position 14 of Bn is not essential for initiating a biologic response.

SECTION OF CLINICAL INVESTIGATION

I. Studies in patients with Zollinger-Ellison syndrome.

The Clinical Investigation Section of the Digestive Diseases Branch has cared for about 170 patients with Zollinger-Ellison syndrome (ZES, a gastrin-producing tumor associated with hypergastrinemia and gastric acid hypersecretion), and smaller numbers of patients with other islet cell tumors.

During the last year, studies have been directed at improving the recognition and diagnosis of ZES, at long-term control of gastric acid hypersecretion with potent antisecretory agents, at developing better methods of localizing the tumor preoperatively, at achieving long-term cure in selected patients and at developing information on the course of patients post curative resection, an area in which very little is now known.

In regard to disease recognition and diagnosis, the studies demonstrate that perforation continued to remain an important presenting symptom in patients, that esophageal disease was much more common in patients with ZES than previously thought, and it was frequently was very difficult to treat. A study of reflux esophagitis and its response to therapy in 122 patients with ZES demonstrated that reflux was not rare, as had been thought previously, but occurred at some time in 61% of patients. When acid outputs were reduced to <10 mEq/h, reflux disease resolved in 2/3 of those affected. However, the other 33% required acid to be lowered to <5 or <1 mEq/h to resolve the disease completely.

In the past, presenting with a complication of peptic ulcer disease was a common form of presentation of ZES but it is generally thought that this is a rare form of presentation today. Our studies demonstrate 10% of our patients present with perforation, frequently jejunal in location and that Billroth operations should be avoided in this situation.

In relation to possible etiologic factors causing the peptic ulceration that occurs in most patients with ZES, our studies in the last year demonstrate that Heliobacter pylori is not a factor, as it is frequently in the case of patients without ZES.

Patients with islet cell tumors such as ZES frequently secrete multiple gastrointestinal hormones and it has been proposed it is important to screen patients regularly for their presence as it may help assess disease extent or location. Our studies demonstrate that additional plasma elevations of gastrointestinal hormones are frequently present (44% had one additional, 18% had two additional hormones besides gastrin); however, routine screening is unnecessary because their presence is clinically not important.

We performed three prospective studies assessing gastrinoma localization methods including examining the value of portal venous sampling (PVS) in 90 cases, the value of ultrasound and a new localization study involving the injection of secretin during arteriography and collecting hepatic venous samples for gastrin concentration. Results show that PVS can be safely done, but should be replaced by the technically easier secretin-angiography study, and that ultrasound, although having a low sensitivity should be retained because it identifies tumors not seen in other studies.

Increasing numbers of patients in the general population are having to take long-term the potent H^+-K^+ ATPase inhibitor, omeprazole for peptic disease. However, this agent causes hypergastrinemia in animals and gastric carcinoid tumors thought secondary to the long-standing hypergastrinemia. Patients with ZES, if not cured, have life-long hypergastrinemia and gastric acid hypersecretion and must keep taking omeprazole indefinitely. Patients with ZES thus are a useful model for assessing the long-term risk of hypergastrinemia in man. Our prospective study of patients with ZES taking omeprazole for up to four years demonstrated that this antisecretory agent did not cause gastric carcinoid tumors in patients with ZES, suggesting the short-term risk is low.

This year we analyzed our ten-year study of the ability to surgically cure 73 patients with ZES. Our data demonstrates that long-term cure was possible in 30% of patients. Furthermore, this study demonstrates the malignancy rate was much higher than previously thought in patients with duodenal primary tumors. Lastly, this study demonstrates with all of the tumor localization studies now available, tumors can now be found in 92% of all patients.

II. Studies of gastric smooth muscle cells.

The regulation of gastrointestinal smooth muscle contraction and relaxation is important in the regulation of motility and is influenced by a number of gastrointestinal hormones and neurotransmitters. However, very little is known about the cellular basis of action of these hormones in muscle cells. In an attempt to directly study the interaction of potentially important gastrointestinal hormones with gastrointestinal muscle, we have utilized methods to study the interaction of various hormones with these cells and the ability of these hormones to cause subsequent changes in biologic activity.

In the last year we have charcterized receptors on dispersed isolated gastric smooth muscle cells for β-adrenergic, opioid and somatostatin-related peptides.

β-adrenergic receptors were of the β_2-adrenergic subtype and mediated a relaxant effect by increasing cAMP. Opioid receptors were of the μ and κ subtypes with none of the δ subtype detected and each of the two subtypes mediated a contractant effect. For each of the two opioid subtypes there was a large degree of receptor spareness. Somatostatin-related peptides inhibited the effects of relaxants, without an effect on contractants by interacting with specific receptors. This inhibitory effect was mediated by pertussis-toxin sensitive G-proteins and involved both inhibition of adenylate cyclase as well as a distal cellular site of action. The natural occurring peptides somatostatin-14 and somatostatin-28 were degraded at markedly different rates by neutral endopeptidases on the smooth muscle cells suggesting this might be important in determining the relative duration of action of these two peptides in vivo.

To assess whether increases in cAMP were essential for the relaxant effects of various peptides on gastrointestinal smooth muscle cell function, the protein kinase A inhibitor R_p-cAMP was used. This agent completely inhibited the ability of exogenous cyclic AMP nucleotides to cause relaxation in a competitive manner, and inhibited the effect of relaxant agents such as CGRP, glucagon or β-adrenergic agents without inhibiting contractants. These results demonstrate activation of protein kinase A was essential for mediating the relaxant action of these peptides.

SUMMARY

Liver Diseases Section

The Liver Diseases Section is currently responsible for seven principal projects.

I. Studies Relating to the Pathogenesis of Hepatic Encephalopathy

The abnormal pattern of visual evoked responses (VERs) in animals with hepatic encephalopathy (HE) due to fulminant hepatic failure (FHF) resembles that induced by drugs which promote GABAergic neurotransmission, including benzodiazepines (BZs). Furthermore, animals with HE due to FHF exhibit increased resistance to drugs that induce convulsions by decreasing GABAergic tone. Ameliorations of HE (both clinical and electrophysiologic) have been induced in animals with FHF by BZ receptor antagonists. Furthermore, Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABAergic tone; (ii) Blokading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE. Increased levels of 1,4-BZs have been demonstrated in the brain of models of HE and humans with FHF. The nature of unidentified BZ receptor ligands present in HE is being sought and BZ receptor ligands with antagonist properties are being screened for optimal properties for use in the management of HE. (E.A. Jones, J. Vergalla, C. Yurdaydin, B.L. Baker, A.S. Basile, P. Skolnick, S.M. Paul).

II. Trials of Therapies for Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to nonsuppurative, presumably autoimmune, destruction of small bile ducts. As some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with PBC. Ten patients with symptomatic PBC are being treated in an open trial of low-dose (15 mg once weekly) oral methotrexate for up to 2 years. Elevated serum IgM and alkaline phosphatase levels decreased significantly and symptom scores for fatigue and pruritus fell by over 50%. Serum ALT levels initially increased but subsequently fell below baseline. Methotrexate therapy was also associated with a substantial decrease in hepatic inflammation. A randomized trial of two doses of methotrexate (7.5 and 15 mg/week) for asymptomatic as well as symptomatic patients with PBC has been initiated (E.A. Jones, J.H. Hoofnagle, N.V. Bergasa, A.M. Di Bisceglie, M.G. Swain, M. Fried, C. Yurdaydin).

III. Studies of Cellular Immune Function in Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the mediation of the hepatobiliary lesion of primary biliary cirrhosis (PBC) is being studied. CD4+, Leu-8+ T cells from patients with PBC, but not from patients with other liver diseases, have been shown to exhibit a defect in their ability to suppress immunoglobulin synthesis by B cells in vitro. Furthermore the proliferative responses of these cells from patients with PBC to mitogenic stimulation was found to be impaired. Exposure of this subpopulation of T cells from patients with PBC to phorbol ester, which activates the protein kinase C pathway, corrects their abnormal function. Thus a defect in the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. In contrast to control patients with non-PBC chronic inflammatory liver diseases, mRNA for IL-1, 2, 4, 5 and 6, INF-γ and TNF-α were not detected in liver biopsies from patients with PBC. These findings suggest that immunologic injury that is not mediated by cytokines plays a major role in disease progression in PBC. (E.A. Jones, M. Shindo, J.H. Hoofnagle, J. Vergalla; non-NIH: S.P. James.

IV. Studies of the Natural History and Treatment of Chronic Type B Hepatitis

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials in which antiviral or immunomodulatory agents are being administered. A randomized control trial of interferon therapy versus no treatment has been completed. Forty-seven patients were entered and all completed follow-up. Eleven of 26 treated with interferon (42%) responded with loss of HBeAg from serum. In addition, pilot studies are examining the effect of one month of corticosteroid pre-treatment followed by interferon therapy for patients who have previously not responded to interferon therapy alone. The effects of ribavirin in patients with chronic hepatitis B is also being studied. (J.H. Hoofnagle, A.M. Di Bisceglie, P. Martin, N.V.Bergasa, J. Korenman, M. Lisker-Melman, B.L. Baker, T-L. Fong, E. A Jones; not NIH: J. Gerin, M. Sjogren).

V. Studies of the Natural History and Treatment of Chronic Non-A, Non-B (Type C) Hepatitis

Patients with well-documented chronic non-A, non-B hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients is available to enable experimental therapies for this disease to be evaluated. Early studies demonstrated that alpha interferon was effective in normalizing serum ALT activities in a majority of cases. This effect was associated with an improvement in liver histopathology (decreased activity of hepatitis). Response to interferon therapy is associated with clearance of hepatitis C viral RNA from serum. In a pilot study of ribavirin therapy 4 of 13 patients with chronic hepatitis C responded with a decrease in serum aminotransferase activities to normal and significant decreases in the titer of HCV RNA in serum. (J.H. Hoofnagle, A.M. Di Bisceglie, N.V.Bergasa, J. Korenman, H.J. Alter, J. Everhart, E.A. Jones; not NIH: Z Goodman, G. Kuo).

VI. Immunological Studies in Chronic Viral Hepatitis

Immunological factors seem to be important in determining the course and outcome of both acute and chronic viral hepatitis. Furthermore, promising therapies for chronic viral hepatitis have profound effects on immune function and sustained responses to therapy may depend largely on restoration of normal immune responsiveness. The role of immunologic mechanisms in determining the course and ultimate outcome of viral hepatitis is being studied. Cytokine mRNAs were determined using a reverse transcriptase (RT) PCR technique in liver biopsies from 14 patients with HBeAg positive chronic hepatitis B and 6 normal subjects (transplantation donors). Only one of the control biopsies was positive for 2 of the 7 mRNAs assayed. In contrast, there was a high prevalence (43-80%) of detectable mRNAs for IL-1, IL-2, IL-4 and IFN-γ in chronic hepatitis B. mRNAs for IL-5, IL-6 and TNF-α were not prevalent in chronic hepatitis B. Quantitative RT -PCR revealed that high levels of mRNA for IL-4 and IFN-γ correlated with severe inflammation, suggesting that the production of IL-4 and IFN-γ may be important in tissue injury in the liver in chronic hepatitis B with active viral replication. (E.A. Jones, J.H. Hoofnagle, A.M. Di Bisceglie, M. Shindo; non NIH: S.P. James).

VII. Studies of the Opiate System in Cholestatic Liver Disease

Pruritus is a common distressing complication of cholestatic liver diseases. The hypothesis that endogenous opiate ligands may contribute to this symptom is suggested by the precipitation of an opiate withdrawal-like syndrome by an opiate antagonist in patients with chronic cholestatic liver diseases and the tendency of exogenously administered opiates to induce pruritus. To test this hypothesis the effects of infusing naloxone in pruritic patients with primary biliary cirrhosis is being assessed using a newly designed device which enables scratching activity to be continuously recorded independent of coarse body movements. Naloxone has been shown to reduce scratching activity substantially in such patients. Further studies of the opiate system in cholestasis revealed that bile duct resection in the rat induces a state of antinociception which can be stereoselectively reversed by naloxone. These findings suggest that in cholestasis there is increased opiatergic tone which could contribute to the pruritus that complicates this syndrome. Long term amelioration of this form of pruritus may be possible by administering an opiate antagonist that is effective when given orally. (E.A. Jones, N.V. Bergasa, T-L. Fong, M. Fried, M.G. Swain, D. Alling, T. Talbot, W. Schmidt).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 53001-21 DDB

PERIOD COVERED
October 1, 1990, to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of Membrane Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jerry D. Gardner	Chief	DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	P.N. Maton	Visiting Scientist	DDB, NIDDK
	S.A. Wank, R. Vinayek, H Frucht	Medical Staff Fellows	DDB, NIDDK
	G. Slimak, D. Metz	Medical Staff Fellows	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	R. Patto	Special Volunteer	DDB, NIDDK
	J. Mrozinski	Chemist	DDB, NIDDK
	Juan Gregory	Intramural Research Training Award	DDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Gastroenterology

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.1	2.1	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The broad categories which are included in the project are: 1) characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 53002-19 DDB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gastrointestinal Hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Gardner	Chief	DDB, NIDI
Others:	R.T. Jensen	Chief, Cell Bio. Sect.	DDB, NIDDK
	P.N. Maton	Visiting Scientist	DDB, NIDDK
	S. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	H.C.V. Chiang, I. Waxman	Medical Staff Fellows	DDB, NIDDK
	S-C. Huang, D. Menozzi, L. Zhang	Visiting Fellows	DDB, NIDDK
	T. Honda	Visiting Assoicate	DDB, NIDDK
	R. Patto	Special Volunteer	DDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Gastroenterology

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project terminated

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53004-19 DDB

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Cyclic Nucleotide Mediated Functions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	J.D. Gardner	Chief	DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	P.N. Maton	Visiting Scientist	DDB, NIDDK
	S.A. Wank, R. Vinayek	Medical Staff Fellows	DDB, NIDDK
	G. Slimak, D. Metz	Medical staff Fellows	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	R. Patto	Special Volunteer	DDB, NIDDK
	J. Mrozinski	Chemist	DDB, NIDDK
	J. Gregory	Intramural Research Training Award	DDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.1	2.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 53100-03 DDB

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Identification and Characterization of Receptors for GI Peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Chief, Cell Biology Section	DDB, NID
Others:	Jerry D. Gardner	Chief	DDB, NIDDK
	S, Wank, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	J.R. Pisegna	Medical Staff Fellow	DDB, NIDDK
	J-M Qian, L-H Wang	Visiting Fellows	DDB, NIDDK
	Y. Kitsukawa	Visiting Fellow	DDB, NIDDK
	S. Mantey	Chemist	DDB, NIDDK
	P. Hildebrand	Special Volunteer	DDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Digestive Diseases Branch

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2.4	2.4	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

These studies involve the identification of receptors for gastrointestinal peptides by developing selective radioligands for specific receptors, specific potent antagonists, and the characterization of receptors using these ligands, antagonists, cross-linking studies, solubilization and eventually structural determination.

PHS 6040 (Rev 1/84) U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53101-03 DDB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Cellular Basis of Action of Gastrointestinal Peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
Others:	Jerry D. Gardner	Chief	DDB, NIDDK
	S. Wank, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	J-M Qian, L-H Wang	Visiting Fellows	DDB, NIDDK
	Y. Kitsukawa	Visiting Fellow	DDB, NIDDK
	S. Mantey	Chemist	DDB, NIDDK
	C. Felley	Special Volunteer	DDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Digestive Diseases Branch

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project includes studies investigating the relationship for various gastrintestinal peptides between receptor occupation to subsequent changes induced in cellular processes, the characterization of the various second messengers including cyclic nucleotides, changes in cellular calcium, breakdown of phosphoinositides, generation of diacylglycerol as well as characterization of more distal steps in cell activation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 53200-02 DDB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Management of Islet Cell Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.N. Maton	Visiting Scientist	DDB, NIDDK
Others:	J.D. Gardner	Chief	DDB, NIDDK
	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	G. Slimak, D. Metz	Medical Staff Fellows	DDB, NIDDK
	J. Pisegna, R. Vinayek	Medical Staff Fellows	DDB, NIDDK
	H. Frucht, V. Fishbeyn	Medical Staff Fellows	DDB, NIDDK
	R. Benya	Medical Staff Fellow	DDB, NIDDK

COOPERATING UNITS *(if any)*

National Cancer Institute, Surgery Branch	J.A. Norton
Radiology Department, Clinical Center	J.L. Doppman

LAB/BRANCH
Digestive Diseases Branch

SECTION
Clinical Investigation Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	2.0	

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies involving patients with islet cell tumors (Zollinger-Ellison Syndrome, insulinoma, VIPoma) are directed to further understanding the pathogenesis of the syndrome and to developing alternative, more effective modes of therapy utilizing both medical and surgical approaches.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53201-02 DDB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Receptors on Gastric Smooth muscle Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.N. Maton	Visiting Scientist	DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	T. Pradhan	Chemist	DDB, NIDDK
	Z-F Gu	Visiting Fellow	DDB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Digestive Diseases Branch

SECTION
Clinical Investigation Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In vitro systems are being used to identify receptors on gastric smooth muscle cells by means of radiolabelled ligands, agonists and antagonists, and to examine the mechanisms by which agonists exert their effects.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53501-17 DDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Studies Relating to the Pathogenesis of Hepatic Encephalopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	B.L. Baker	Research Associate	LDS, NIDDK
	M.G. Swain	Guest Researcher	LDS, NIDDK
	C. Yurdaydin	Visiting Associate	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK

COOPERATING UNITS *(if any)*

Laboratory of Neuroscience, NIDDK (P. Skolnick and A.S. Basile)
Mental Health Intramural Research Program, NIMH (S.M. Paul)

LAB/BRANCH
Digestive Diseases Section

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER.
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Rabbits and rats with HE due to FHF exhibit increased resistance to the convulsive effects of bicuculline (a GABA receptor antagonist) and 3-mercaptopropionic acid (an inhibitor of GABA synthesis). Both clinical and electrophysiologic (VER waveform) ameliorations of HE, have been induced in animals with FHF by benzodiazepine (BZ) receptor antagonists. Furthermore, spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. In addition a BZ receptor antagonist reversed the hypersensitivity of HE rabbit neurons to depression by a GABA agonist. The functional status of the chloride ionophore of the GABA/BZ receptor complex has been shown to be normal in a rat model of HE due to FHF. Radioligand binding to BZ receptors, determined autoradiographically, was decreased in thin unwashed sections from HE rabbit brains. Purification and characterization of HE rat brain extracts revealed the presence of reversible, competitive, heat and protease stable BZ receptor ligands with agonist properties. Two of these ligands have been chemically characterized as the 1,4-BZs diazepam and N–desmethyldiazepam. The concentrations of these compounds were 2–9 fold greater in HE rat brain than control brain. Overall, these findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) Endogenous BZ receptor agonists probably contribute to HE. Current research using animal models is concerned with identifying other BZ receptor ligands present in HE and identifying BZ receptor antagonists that have optimal properties for the management of HE.

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK, 53503-16 DDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunologic Studies of Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director	DDN, NIDDK
	J. Vergalla	Chemist	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	M. Shindo	Guest Researcher	LDS, NIDDK

COOPERATING UNITS *(if any)*

Division of Gastroenterology, University of Maryland at Baltimore (S.P. James)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Primary biliary cirrhosis (PBC) appears to be a model autoimmune disease. Abnormal immune mechanisms are being studied in this disease, but so far a disease-specific immunologic deficit has not been defined with certainty. To determine whether previously described abnormalities of lymphocyte function in PBC might be due to altered function of immunoregulatory T cell subpopulations, phenotypic and functional characteristics of T cells that have the CD4 antigen detectable (by monoclonal antibody) on their surface were examined. Patients with PBC were found to have normal numbers of CD4+, Leu-8+ T cells, but, in contrast to patients with other liver diseases, suppression of immunoglobulin synthesis and mitogen-stimulated proliferation mediated by this subpopulation of T cells were defective. These defects in the function of CD4+, Leu-8+ T cells in patients with PBC were corrected by phorbol ester suggesting that abnormal function of the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. In contrast to control patients with non-PBC chronic inflammatory liver diseases, mRNA for IL-1,2,4,5 and 6, IFN-γ and TNF-α were not detected in liver biopsies from patients with PBC. While these findings do not exclude a role for cytokines in the mediation of bile duct lesions in PBC, they suggest that immunologic injury that is not mediated by cytokines plays a major role in disease progression in PBC.

PHS 6040 (Rev 1/84)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53509-12 DDB

PERIOD COVERED
October 1, 1990 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies of the Natural History and Treatment of Chronic Type B Hepatitis

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator*) (*Name, title, laboratory, and institute affiliation*)

PI:	J.H. Hoofnagle	Director, DDDN	NIDDK
Others:	E.A. Jones	Chief	LDS, NIDDK
	A.M. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	T.L. Fong	Medical Staff Fellow	LDS, NIDDK
	M. G. Swain	Guest Researcher	LDS, NIDDK
	M. W. Fried	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (*if any*)

Georgetown University, Washington, D.C. (J. Gerin)
Walter Reed Army Institute of Research, Washington, D.C. (M. Sjogren)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials of antiviral and/or immunomodulatory agents. Several agents have been evaluated for antiviral effect, including alpha interferon, dideoxyinosine (DDI) and ribavirin.

A randomized, controlled trial to reevaluate the effects of alpha interferon alone (given at a dose of 10 million units three times weekly) has been completed in 47 patients with chronic hepatitis B. Loss of hepatitis B e antigen (HBeAg) and HBV DNA occurred by 6 mos in 11 of 26 (42%) treated but in only 1 of 21 (5%) control patients ($p=.003$). These findings together with results of previous studies confirm that interferon is effective in inducing a remission in selected patients with chronic hepatitis B.

A pilot study of DDI has been completed in 7 patients with chronic hepatitis B. Although DDI was well tolerated, it did not appear to have significant antiviral action against hepatitis B.

A pilot study of ribavirin therapy is currently underway in patients with chronic hepatitis B. Thirteen patients have been entered into this study and 2 have completed 6 mos of therapy so far. It appears that ribavirin inhibits hepatitis B replication, as most patients have shown a fall in serum levels of HBV DNA polymerase, by approximately 50%. However, no patients have yet cleared HBeAg or HBV DNA from serum. This study will be continued until 18 patients have been treated.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK, 53510-11 DDB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of the Natural History and Treatment of Chronic Non-A, Non-B (Type C) Hepatitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A.M. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	T.L. Fong	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS *(if any)* NIH Blood Bank (H.J. Alter, J.K. Shih), Armed Forces Institute of Pathology, Washington, D.C. (Z. Goodman), Chiron Corporation, Emeryville, CA (M. Houghton, G. Kuo), Laboratory of Infectious Diseases, NIAID (S.M. Feinstone, R.H. Purcell).

LAB/BRANCH
Digestive Diseases Branch

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3	PROFESSIONAL: 2	OTHER: 1

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with well-documented chronic non-A, non-B hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients are available to evaluate experimental therapies. Previous studies have shown that alpha interferon therapy has a short-term beneficial effect in approximately 50% of patients during treatment and that the beneficial response is sustained in approximately 20% after stopping interferon. A pilot study of ribavirin therapy has been conducted in 14 patients with chronic hepatitis C. Serum aminotransferases decreased during therapy in all patients and became normal in 5 (36%). Serum levels of HCV RNA also decreased significantly during therapy.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 53511-11 DDB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Trials of Therapies for Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	A. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	M.G. Swain	Guest Researcher	LDS, NIDDK
	M. Fried	Medical Staff Fellow	LDS, NIDDK
	C. Yurdaydin	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

Digestive Diseases Branch

LAB/BRANCH

SECTION
Liver Diseases Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1	PROFESSIONAL: 1	OTHER:

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary biliary cirrhosis (PBC) is an autoimmune disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative inflammatory destruction of small intrahepatic bile ducts. Because some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with PBC. In a randomized controlled trial of chlorambucil therapy, treatment with the drug was associated with a decrease in the rate of increase of serum bilirubin, normalization of elevated serum IgM levels and an improvement in inflammatory cell infiltration in liver biopsies. These encouraging findings have prompted a search for safer and more effective immunosuppressive drugs with less carcinogenic potential than chlorambucil. One promising drug of this type is methotrexate. Ten patients with symptomatic PBC were admitted to an open trial of low-dose (15 mg) once weekly oral methotrexate. Two patients with advanced (stage III-IV) disease dropped out after 4-5 months. The remaining 7 were followed for 8-24 months. Methotrexate therapy in these patients was associated with significant decreases in elevated serum levels of IgM and alkaline phosphatase and a more than 50% decrease in symptom scores for fatigue and pruritus. Serum ALT levels initially increased (4 and 8 mo.) but subsequently fell to 10% below baseline. In 4 patients, who had liver biopsies before and after one year of treatment, methotrexate was associated with a substantial decrease in hepatic inflammation. These encouraging findings have prompted the initiation of a randomized trial of two doses of methotrexate (7.5 and 15 mg/week) for asymptomatic as well as symptomatic patients with PBC.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53514-07 DDB

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunological Studies in Chronic Viral Hepatitis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	M. Shindo	Guest Researcher	LDS, NIDDK

COOPERATING UNITS *(if any)*

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SECTION

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CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Immunological factors seem to be important in determining the course and outcomeof both acute and chronic viral hepatitis. Furthermore, promising therapies for chronic viral hepatitis have profound effects on immune function and sustained responses to therapy may depend largely on restoration of normal immune responsiveness. The role of immunologic mechanisms in determining the course and ultimate outcome of viral hepatitis is being studied. Cytokine mRNAs were determined using a reverse transcriptase (RT) PCR technique in liver biopsies from 14 patients with HBeAg positive chronic hepatitis B and 6 normal subjects (transplantation donors). Only one of the control biopsies was positive for 2 of the 7 mRNAs assayed. In contrast, there was a high prevalence (43-80%) of detectable mRNAs for IL-1, IL-2, IL-4 and IFN-γ in chronic hepatitis B. mRNAs for IL-5, IL-6 and TNF-α were not prevalent in chronic hepatitis B. Quantitatiave RT-PCR revealed that high levels of mRNA for IL-4 and IFN-γ correlated with severe inflammation, suggesting that the production of IL-4 and IFN-γ may be important in tissue injury in the liver in chronic hepatitis B with active viral replication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK, 53516-01 DDB

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Studies of the Opiate System in Cholestatic Liver Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI	E. A. Jones	Chief	LDS, NIDDK
Others:	N. V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	J. Vergalla	Chemist	LDS, NIDDK
	M.G. Swain	Medical Staff Fellow	LDS, NIDDK
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COOPERATING UNITS *(if any)*
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SECTION
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TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
1.5	1	.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new system for continuously quantitating scratching activity, independent of gross limb movements, has been devised. This system permits objective quantitative studies of the effectiveness of therapies for the pruritus of cholestasis to be undertaken. Using this system naloxone infusions have been shown to decrease a scratching activity index by about 50% in patients with pruritus due to primary biliary cirrhosis, suggesting that the opiate system may be implicated in the mediation of the pruritus of cholestasis . Support for the hypothesis that the status of the opiate system is altered in cholestasis has been provided by showing that rats with acute cholestasis due to bile duct resection exhibit antinociception (as assessed by the tail flick assay) and that this pathophysiologic state can be stereoselectively reversed by naloxone. Furthermore, increased levels of total opioid activity and methionine enkephalin have been demonstrated in plasma in this model of cholestasis. These findings are consistent with the syndrome of cholestasis being associated with increased opiatergic tone mediated by endogenous opioids. As opiates, such as morphine, are known to induce pruritus, the mechanism of the pruritus of cholestasis could be increased opiatergic tone. A double blind trial of naloxone infusions for the pruritus of chronic cholestasis is nearing completion. An open trial of the orally bioavailable opiate antagonist nalmefene for this complication of cholestasis is also in progress. In six patients oral nalmefene was associated with a clinical amelioration of pruritus in all, an improvement in visual analogue scores in 5 and a decrease in scratching activity index in 4. In additional studies of the opiate system in cholestasis, bile duct resected rats have been shown to exhibit a naloxone-reversible decrease in the ACTH response to stress.

ANNUAL REPORT OF THE
MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The MCNEB continues basic and clinical investigations in the areas of molecular regulation and neuroendocrinology (Molecular Regulation and Neuroendocrinology Section, Bruce D. Weintraub, Chief); and growth and development (Growth and Development Section, Matthew M. Rechler, Chief). The Branch has had many visiting fellows and associates, as well as international collaborations with the University of Milan, Italy; University of Marseilles, France; Karolinska Institute, Sweden; Postgraduate School of Obstetrics and Gynecology, University of Auckland, New Zealand; University of Naples, Italy; Department of Medicine, University of Gothenburg, Sweden; University of Madrid, Spain. .

Dr. Weintraub was honored as a Visiting Professor at the Johns Hopkins Hospital, UCLA Medical Center (Boris Katz Professor), and Philadelphia Endocrine Society. He was also chosen as a plenary speaker at the Latin American Thyroid Association Meeting in Sao Paulo, Brazil, the UCLA Symposium on Thyroid Hormone Action in Tamarron, Colorado, and the International Thyroid Meeting in The Hague, Netherlands.

I. THYROTROPIN, THYROTROPIN-RELEASING HORMONE AND THYROID HORMONE: MOLECULAR BIOLOGY, REGULATION, ACTION, AND PATHOPHYSIOLOGY.

A. Large Scale Production of Recombinant Human Thyrotropin (TSH) and Its Analogues: Clinical Trials in Patients with Thyroid Cancer

We have previously expressed recombinant human (rh)TSH after transient or stable transfection of α and hTSH-β-minigenes into various mammalian cell lines including Chinese hamster ovary cells. Carbohydrate compositional analysis of recombinant thyrotropin showed it to be highly sialylated with no GalNAc and therefore is lacking terminal sulfate moieties, which are both normally present in pituitary–derived TSH. Thus, this is a novel glycoform of hTSH containing only sialic acid–terminating oligosaccharides and is closer to hypothyroid hTSH. Immunologic activity of rhTSH was about two–fold lower and receptor–binding activity was similar to that of the standard pituitary TSH. Removal of sialic acid did not alter the immunologic and receptor–binding activities of rhTSH. The rhTSH was fully active in two different in vitro bioassays. However, rhTSH was about three–fold and eight–fold less potent in adenylyl cyclase stimulation in bovine thyroid membranes and cAMP production in FRTL–5 cells respectively than pituitary TSH. The lower in vitro biological activity may be due to its highly sialylated oligosaccharide chains. The lower potency in the above assays was reversed by sialic acid removal from rhTSH and the asialo–rhTSH showed potency similar to pituitary hTSH in the above two assays. The rhTSH had a 2.6–fold lower metabolic clearance rate than pituitary TSH, due to a decrease in the rapid as well as slow phases of clearance. After sialic acid removal, the rhTSH was cleared much faster than pituitary hTSH, showing that its longer plasma half–life was due to its higher sialylation.

Currently, milligram amounts of rhTSHG are being produced in a hollow fiber bioreactor system. Novel purification methods, including affinity chromatography, are being utilized to obtain sufficient amounts for further oligosaccharide characterization and biologic studies. A variety of analogues of hTSH are also being purified including glycosylation variants as well as forms with altered protein structure, produced by site-directed mutagenesis.

Through a Cooperative Research and Development Agreement with the Genzyme Corporation (Boston), the company and NIH are producing recombinant human TSH for clinical studies in patients with thyroid cancer. This product is expected to stimulate uptake of radioactive iodide for goiter diagnostic and therapeutic purposes and to obviate the need for performing uptake studies in hypothyroid patients. An Investigational New Drug Application has been submitted to the Food and Drug Administration, and clinical trials are expected to begin in late 1991. The NIH Clinical Center will be involved in all phases of these clinical trials, which will begin after an appropriate protocol has been approved by the Institutional Review Board. Four other medical centers in the United States will also participate in Phase I studies.

.... N. R. Thotakura, L. Joshi, M. Szkudlinski, J. E. Palmer, B. D. Weintraub

B. Role of carbohydrate chains of TSH subunits in the hormone's action:

The relative importance of the carbohydrate chains in individual subunits of bTSH was studied using differentially deglycosylated TSH. The α and β subunits of bTSH were deglycosylated with endoglycosidase F and were recombined in various combinations to produce native TSH ($\alpha\beta$), deglycosylated TSH (dgαdgβ) or hybrid molecules (αdgβ and dg$\alpha\beta$). The in vitro bioactivities of these derivatives were studied using bovine thyroid membrane adenylyl cyclase assay and stimulation of cAMP and growth in FRTL-5 cells. In the adenylyl cyclase assay, deglycosylation of either the α or both subunits of bTSH reduced the V_{max} to 13% of $\alpha\beta$, whereas removal of carbohydrate from β subunit alone had less pronounced effect (V_{max}, 50% of $\alpha\beta$). In both the FRTL-5 assays, removal of carbohydrate from the α but not β subunit of bTSH caused a 2-3 fold reduction in the potency. The adenylyl cyclase assay was more sensitive to carbohydrate removal than the FRTL-5 assay in that the less active derivatives stimulated cAMP in the latter assay to near maximal levels but at higher concentrations. These results indicate that the carbohydrate chains in both subunits of TSH are important in hormone action, removal of carbohydrate from the α subunit showing more pronounced effects. We also demonstrate that deglycosylation causes variable effects in different bioassays. Such studies will be important in developing new glycosylation variants of rhTSH for clinical use in patients.

.... N. R. Thotakura, B. D. Weintraub

C. Thyroid Hormone Inhibitory Domains Within the Human Thyrotropin Beta (hTSH-β) Gene.

The process of understanding the varied effects of T3 has only recently progressed from the physiologic level to T3 regulation of gene expression. T3 stimulation of rat growth hormone production has been found to be mediated by interaction between thyroid hormone, its receptor, and a grouping of three putative binding sites. However, mechanisms of T3 inhibition of gene expression have not been elucidated.

The first exon of the human TSHβ gene has been demonstrated in our laboratory to contain a major thyroid hormone inhibitory element and we examined the TSH gene as a model of negative T3 transcriptional control. A variety of DNA binding techniques and functional studies were used to identify major regions within the TSHβ first exon necessary for thyroid hormone action. Functional studies were performed by a scanning mutation analysis using transient transfection of human TSHβ expression vectors containing 5 bp cassette mutations within the first exon of the human TSHβ gene. Various -1200 to +37 fragments of the human TSHβ gene containing consecutive five deoxythymidine substitution mutations (-3 to +7, +13 to +17, +18 to +22, +23 to +27, +28 to +32, and +33 to +37) were placed within a luciferase reporter plasmid and transiently transfected into human embryonal kidney cells using the calcium phosphate precipitation method. Identical constructs were stably cloned within GH3 cells. Both transient and stable transfection studies identified two non-contiguous regions of the first exon which are both required for T3 inhibition. Mutation of the 3' end of the first exon showed loss of inhibition by T3 between +28 and +37 (0 to 16% of wild type control) in both 293 cells and GH3 cells. Mutations at the 5' end of the first exon from +3 to +22 in 293 cells and between +3 and +27 in GH3 cells resulted in loss of T3 inhibition (0 to 22% of wild type control). Mutations from +23 to +27 in 293 cells and +18 to +27 in GH3 cells had no effect on T3 inhibition.

The avidin-biotin complex DNA-binding assay, a very sensitive measure of DNA-protein interactions, was used to study binding of thyroid hormone receptor to the first exon. Identical 5 bp cassette mutations as those used in the functional studies were constructed and bound to ^{35}S-labeled c-erbA-β. Mutations -3 to +2, +3 to +7, and +8 to +12 resulted in significant loss of c-erbA-β binding (5.5%, 27.5% and 55.2%). Mutations from +18 to +32 did not reduce c-erbA-β binding. Mutations in the 3' end of the first exon; from +23 to +27, +33 to +37, and +38 to +43 bp resulted in 60.6%, 87% and 66.7% c-erbA-β binding, respectively, compared to wild type levels. The addition of mouse thyrotropic tumor extract did significantly alter binding. These binding experiments corroborate the functional studies, again identifying two noncontinuous regions of DNA interaction with thyroid hormone receptor. Moreover, the binding data suggest that the 5' region may interact more strongly with c-erbA-β. To explore this avenue further, DNA affinities of the two domains from c-erbA-β was measured using the ABCD assay. Three fragments of the first exon were synthesized; -12 to +43, -12 to +19 and +18 to +43, and bound in varying concentrations to labeled c-erbA-β. Scatchard analysis revealed the 5' region (-12 to +19) to be of higher affinity than the 3' region (+18 to +43) with affinities of 7 nM and 14 nM, respectively. Moreover, gel mobility shift assay using radiolabeled -12 to +43 bp TSHβ probe and *in vitro* translated c-erbA-β revealed a single protein-DNA complex that was successfully competed by excess stimulatory thyroid hormone response element from the rGH but not by a cold +18 to +43 bp fragment of human TSHβ. This study further indicates that the 5' binding domain has a higher affinity for c-erbA-β than the 3' region.

In summary, functional experiments using two cell systems and a variety of DNA binding assays indicate two regions of the first exon of the hTSH-β gene that are required for c-erbA-β transcriptional control. The 5' region extending from +3 to +17 bp has a higher affinity for c-erbA-β and both regions are necessary for T3 action. Methylation interference studies pinpoint deoxyguanosine residues at +1, +2, +3 and +12 as intimately involved in c-erbA-β interaction with the first exon.

. . . . D. L. Bodenner, F. E. Wondisford, B. D. Weintraub

D. Pit-1 as a Mediator of TRH and cAMP Stimulation of Human TSH-β Gene Transcription

Previous work from our laboratory has demonstrated that a fragment of the TSHβ promoter from -128/+8 will support a stimulatory effect of both TRH and cAMP when linked to the bacterial reporter gene CAT and expressed transiently in the rat pituitary cell line GH3. Deletion analysis of this fragment indicated that two independent regions of this promoter are required for the full effect of both agents: an upstream region -128/-60 which contains elements competent to bind the pituitary-specific transcription factor, Pit-1, and a small sequence +3/+8 which overlaps a consensus half-site for T3 receptor binding and, we have noted, a strong homology with the binding site for AP-1. This latter activity is composed of dimers of members of the fos and jun family of proto-oncogenes and for a number of genes is the mediator of the transcriptional response to phorbol esters. Moreover, we have recently observed that the hormonal response of the TSHβ promoter is enhanced by maintenance of GH3 cells in serum-free medium prior to transfection and hormone-treatment.

(a) Phorbol esters stimulate human TSHβ gene expression.
The promoter fragment -128/+8 mediates a 5-7 fold stimulatory response in transient transfection assays in GH3 cells which is maximal at 20 nM PMA and localized to the upstream region as defined by the studies of TRH and cAMP stimulation. Despite overlap with the AP-1 site, bases +3/+8 do not contribute to this response under conditions of pretreatment with serum-free media which is expected to minimize endogenous AP-1 activity.

(b) The TRH stimulation of TSHβ gene transcription is mediated in part by protein kinase C. Down-regulation of PKC activity by pretreatment of cells with supramaximal doses of PMA (200 nM) for 48 hrs prior to TRH treatment results in an 60-80% reduction in TRH stimulation. Promoter fragments containing only the downstream region -28/+8 however mediate a TRH response under conditions in which a PMA response is not seen consistent with the existence of PKC-independent pathways of TRH stimulation.

(c) The transcriptional activity AP-1 is an activator of TSHβ gene expression. Cotransfection of GH3 cells with fos and jun expression vectors increases TSH-128/+8 promoter-dependent CAT activity by 2-3 fold, an effect localized primarily to downstream regions containing an intact AP-1 binding site homology.

As previously observed in GH3 and 293 cells, treatment with forskolin (adenylate cyclase activator) or PMA, or cotransfection of the Pit-1 expression vector did not activate the expression of the transfected hTSHβ -128/+8 CAT construct in HeLa cells. However, a 10-fold stimulation was observed by the same construct when the cells were cotransfected with the Pit-1 expression vector in conjunction with the forskolin or PMA treatment. In contrast, this treatment could not overcome the effect of the repressor element in the -1200/+8 CAT construct. Since the sequences from -1 to +6 (TGGGTCA) bear homology to the AP-1 site (TGAGTCA) and the cAMP response element (TGACGTCA), we next examined whether this element could mediate the forskolin or PMA stimulation. When the plasmids containing mutations of this element in the hTSHβ -128/+8 CAT gene were cotransfected with the Pit-1 expression vector into HeLa cells, the induction by forskolin or PMA was completely abolished. As expected, deletion of the Pit-1 binding sites from the hTSHβ gene also abolished the observed induction further confirming the role of Pit-1 in mediating the forskolin or PMA stimulation. Taken together, these data indicated that both Pit-1 and the factors which bind to the TGGGTCA element appear to interact directly or indirectly in mediating the forskolin or PMA induction of the hTSHβ gene.

. . . . J. H. McClaskey, M. K. Kim, M. Blackman, B.D. Weintraub

E. Transgenic Tumors of the Mouse Thyrotrope

The study of TSH regulation and thyrotropic specific cell factors has been hampered by the lack of a thyrotropic specific cell line culture. Current investigations are limited to the pituitary cell dispersions or passage of rat thyrotropic tumors by muscle injection. Attempts to establish immortalized thyrotropic tumor cells have included culture of human TSH-β producing tumors, immortalized pituitary cell cultures, and transgenic animals containing tumor-promoting antigens coupled to various α or β segments of the TSH gene. All have been unsuccessful to date. Two lines of transgenic mice containing a segment of the human TSH-β gene coupled to SV40 viral DNA coding for the tumor promoting T-antigen have been developed in our laboratory. These animals produce tumors restricted to the pituitary after nine to fourteen months of life.

An approximately 4 kb segment of the human TSHβ gene encompassing the 5' regulatory region and extending into the first intron was ligated to the large segment of the SV40-T antigen and injected into mouse germ cells. Impregnation resulted in six animals containing the desired transgene. Two animals survived with Southern blot screening demonstrating 2 and 7 copies of the SV40-T antigen present, respectively. Mating with wild type animals was successful with approximately 20-30% of progeny demonstrating heterologous incorporation of the transgene. Three generations of animals have demonstrated no loss in copy number or frequency of transmission. Ten first generation animals of #5 were sacrificed after 9 months of life and 3 mm pituitary tumors were identified at necropsy in two animals without tumors present in other organs. Histologic examination of the tumors revealed one to be highly invasive, extending into and beyond surrounding bone with lacy, eosinophilic cytoplasm and mitotic figures present. The second tumor was restricted to the sella but felt of high grade with dense basophilic nuclei, scant cytoplasm and numerous mitotic figures identified. Both tumors were hemorrhagic. Culture of the tumors was unsuccessful but T-antigen RNA expression was restricted to the tumor and not found in other organs. 52 second generation animals were recently sacrificed between 9 and 12 months of life yielding 4 additional transgenic tumors. All 4 tumors were cultured using pituitary and endothelial growth factors. Three weeks after harvest, a relatively pure culture of very large, flat polygonal cells have been obtained. Analysis of the culture media for TSH both in TRH stimulated and unstimulated flasks is underway as well as isolation and identification of mRNA for SV40-T antigen and TSH-β.

In summary, our laboratory has been the first to develop pituitary tumor-producing transgenic mice containing a large segment of the human TSH-β gene coupled to the tumor-promoting SV40 T-antigen gene. The development of a cell line of these presumable thyrotropic tumors will be invaluable in the study of cell specific factors and expression of the TSH-β gene.

. . . . D.L. Bodenner, F. E. Wondisford, B. D. Weintraub

F. Mutations of the Beta Thyroid Hormone Receptor Gene in Generalized Thyroid Hormone Resistance.

Generalized thyroid hormone resistance (GTHR) is a disorder of thyroid hormone action characterized by elevated free thyroid hormones and TSH, and inappropriate clinical and biochemical signs of euthyroidism or hypothyroidism. It is usually transmitted as a familial syndrome with an autosomal dominant pattern of inheritance. We have previously shown that in a kindred, A, the gene for GTHR is tightly linked to one of the two known thyroid hormone receptor genes, c-erbAβ, on chromosome 3. To study the role of the thyroid hormone receptor gene in this syndrome, we have conducted further linkage analyses in two other kindreds, B and D, with differing degrees of thyroid hormone insensitivity. There was also linkage between c-erbAβ and GTHR loci in these two kindreds, and the combined maximum lod score for all three kindreds at a recombination fraction of 0 was 5.77. We investigated the defect in c-erbAβ in Kindred A by sequencing the T3-binding domain in the 3'-region of fibroblast c-erbAβ cDNA and leukocyte c-erbAβ genomic DNA. A base substitution, cytosine to adenine, was found at cDNA position 1643 which altered the proline codon to a histidine codon. This base substitution was only found in one allele of affected members of Kindred A, as expected for a dominant disease, and was not found in unaffected members, in two other Kindreds, or in 92 random c-erbAβ alleles. Because of this absolute linkage with the abnormal phenotype, and the fact that the mutation is predicted to alter the secondary structure of the crucial T3-binding domain of the c-erbAβ receptor it was likely to represent a c-erbAβ mutation responsible for GTHR in kindred A.

Recently we have discovered novel and completely different point mutations of the thyroid hormone β receptor in 14 other kindreds. We are currently employing new DNA mismatch and chemical cleavage techniques to screen rapidly for mutations in other families that we have studied previously at NIH.

.... A. J. Mixson, P. Hauser, S. R. Lasky, B.D. Weintraub

G. Functional Characterization of Mutant Receptors.

We functionally characterized five mutant T3 receptors. The T3-binding affinity was assessed using in vitro translated protein whereas the transcriptional capacity was tested in a cotransfection system. The T3 affinity of all the mutant receptors studied was reduced or undetectable and correlated well with the degree of impairment of the transactivating function in HeLa cells. Two mutant receptors with undetectable ligand affinity showed no transcriptional activity whereas the three other mutants characterized by a 2-, 5- and 7-fold reduction in T3 affinity required 5-, 10- and 15-fold higher T3 concentrations for half-maximal activity in the cotransfection assay, respectively. All of the mutant hTRβ1 studied were able to inhibit the function of normal hTRβ1 in activating a palindromic positive T3 response element (TRE). In the partially functional mutants this dominant negative effect could be completely reversed by increased T3 concentrations. The dominant negative potency did not depend on the type of TRE used; mutant hTRβ1 were able to inhibit normal receptor function to the same degree on a dimer-permissive palindromic TRE as on a non-dimer-permissive inverted repeat of two identical half-sites separated by five spacer bases.

.... C. A. Meier, S. C. Ransom, B. D. Weintraub

II. Insulin-like growth factors

We have continued our studies of the insulin-like growth factors (IGFs), their receptors and binding proteins to understand the physiological and pathological role of the IGFs. IGF-I and IGF-II in plasma and other extracellular fluids occur complexed to IGFBPs, a family of at least 6 proteins that are thought to modulate IGF actions. We believe that a major component of this regulation occurs at the levels of IGFBP synthesis in different tissues. The IGFBP-2 gene is expressed at higher levels in fetal than in adult rat tissues, especially liver, and persists in adult choroid plexus. IGFBP-2 mRNA is increased in liver in diabetes, fasting and growth hormone deficiency. We have isolated and characterized clones encoding the rat IGFBP-2 gene. Its promoter region lacks a TATA box, but contains potential cis regulatory elements for insulin regulation and liver-specific expression. Transcription of the IGFBP-1 gene is increased in diabetic rat liver, and decreased after insulin treatment. IGFBP-1 mRNA also is increased in fetal rat liver after maternal food deprivation. We have studied the regulation of IGF PB-1 gene expression in a model system, the well-differentiated H4-II-E rat hepatoma cell line. In H4-II-E cells, IGFBP-1 gene transcription is increased by the synthetic glucocorticoid dexamethasone, and rapidly (<20 min) decreased by physiological concentrations of insulin.

The mammalian IGF-II/Mannose-6-phosphate receptor is a bifunctional receptor that binds IGF-II and proteins containing mannose-6-phosphate recognition markers (such as lysosomal enzymes). Chick embryo fibroblasts contain a primitive form of this receptor. It is the same size as and immunonogically related to the mammalian IGF-II/Man 6-P receptor, binds and internalizes lysosomal enzymes, but does not bind IGF-II.

.... M. M. Rechler, A. L. Brown, J. A. Romanus, L. Tseng, G. T. Ooi, A. Mancini, P. Pioli, Y. Boisclair, Y. W.-H Yang, D. E. Graham, L. Bach

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1 DK 55000-19 MCNE

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biosynthesis, Glycosylation, and Action of Thyrotropin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. D. Weintraub	Chief	MCNEB, NIDDK
Others:	N. R. Thotakura	Visiting Scientist	MCNEB, NIDDK
	J. E. Palmer	Biologist	MCNEB, NIDDK
	M. Szkudlinski	Visiting Fellow	MCNEB, NIDDK

COOPERATING UNITS *(if any)*
None

LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch

SECTION Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.5
PROFESSIONAL: 2.5
OTHER: 0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recombinant human thyrotropin has been produced after transient or stable transfection of alpha and hTSH-beta minigenes into various mammalian cell lines, including Chinese hamster ovary cells. Carbohydrate composition analysis shows that the recombinant TSH has more sialic acid than conventional pituitary TSH, which delays its metabolic clearance rate. Large scale production of recombinant TSH has been achieved by employing a hollow fiber bioreactor system as well as novel methods of purification. Various analogues of TSH have also been produced by modifying glycosylation or protein structure using site-directed mutagenesis. The specific role of carbohydrate in the action of TSH has been elucidated by use of differentially glycosylated subunits.

Through a Cooperative Research and Development Agreement with the Genzyme Corporation (Boston), the Company and NIH are producing recombinant human TSH for clinical studies in patients with thyroid cancer. Approval of the Investigational New Drug Application and the commencement of clinical trials at NIH and four other medical centers is expected in late 1991.

U.S. GOVERNMENT PRINTING OFFICE. 1991 0-164-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1 DK 55002-11 MCNE

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Biology of Pituitary Glycoprotein Hormones and Hypothalamic Releasing Hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. D. Weintraub	Chief	MCNEB, NIDDK
Others:	J. H. McClaskey	Senior Staff Fellow	MCNEB, NIDDK
	D. L. Bodenner	Medical Staff Fellow	MCNEB, NIDDK
	Myung K. Kim	Senior Staff Fellow	MCNEB, NIDDK
	M. Blackman	IPA	MCNEB, NIDDK

COOPERATING UNITS *(if any)*
Dr. Fredric E. Wondisford, Case Western Reserve School of Medicine, Cleveland, Ohio

LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch

SECTION Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3.5 PROFESSIONAL: 3.5 OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have cloned the human thyrotropin beta subunit gene and have localized a major thyroid hormone inhibitory element in the first untranslated exon of this gene using a combination of functional and binding methods. This inhibitory element appeared to be comprised of two distinct half-sites, one located at the 5' end of the exon and another toward the 3' end of the exon. These various results suggest that thyroid hormone receptors binding to at least two sites in the first exon act in conjunction to mediate T3 inhibition of TSH-beta expression. We have also demonstrated that a pituitary-specific transcription factor, Pit-1, mediates both the cyclic AMP as well as the thyrotropin-releasing hormone (TRH) induction of hTSH-beta gene transcription. This is the first example of a factor known to be important in cell-specific expression also mediating hormonal regulation of pituitary gene transcription. Finally, transgenic tumors have been established using constructs of the 5'-flanking region of the human TSH-beta gene fused to the SV40 T-antigen. From such tumors, thyrotropic cell lines have been established which should be of great value in the study of TSH-beta gene expression.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK55006-18 MCNE

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. This must fit on one line between the borders.)*

Insulin-like Growth Factors

PRINCIPLE INVESTIGATOR *(List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	M.M. Rechler	Chief, GDS	MCNEB, NIDDK
Others:	A.L. Brown	Sr. Staff Fellow	MCNEB, NIDDK
	J.A. Romanus	Chemist	MCNEB, NIDDK
	L. Tseng	Chemist	MCNEB, NIDDK
	G.T. Ooi	Visiting Fellow	MCNEB, NIDDK
	A. Mancini	Courtesy Associate	MCNEB, NIDDK
	P. Pioli	Courtesy Associate	MCNEB, NIDDK
	Y. Boisclair	Guest Researcher	MCNEB, NIDDK
	Y.W.-H. Yang	Sr. Staff Fellow	MCNEB, NIDDK
	D.E. Graham	Microbiologist	MCNEB, NIDDK
	L. Bach	Visiting Associate	MCNEB, NIDDK

COOPERATING UNITS *(if any)* University of California-Riverside (D.S. Straus); MB NCI (S.P. Nissley); LBM NIDDK (A. R. Robbins)

LAB/BRANCH

Molecular, Cellular & Nutritional Endocrinology Branch

SECTION

Growth & Development Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	6.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ **(a) Human subjects** ☐ **(b) Human tissues** ☒ **(c) Neither**
  ☐ **(a1) Minors**
  ☐ **(a2) Interviews**

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued our studies of the insulin-like growth factors (IGFs), their receptors, and binding proteins (IGFBPs) to understand the physiological and pathological role of the IGFs. IGF-I and IGF-II in plasma and other extracellular fluids occur complexed to IGFBPs, a family of at least 6 proteins that are thought to modulate IGF actions. We believe that a major component of this regulation occurs at the level of IGFBP synthesis in different tissues. The IGFBP-2 gene is expressed at higher levels in fetal than in adult rat tissues, especially liver, and persists in adult choroid plexus. IGFBP-2 mRNA is increased in liver in diabetes, fasting and growth hormone deficiency. We have isolated and characterized clones encoding the rat IGFBP-2 gene. Its promoter region lacks a TATA box, but contains potential cis regulatory elements for insulin regulation and liver-specific expression. Transcription of the IGFBP-1 gene is increased in diabetic rat liver, and decreased after insulin treatment. IGFBP-1 mRNA also is increased in fetal rat liver after maternal food deprivation. We have studied the regulation of IGFBP-1 gene expression in a model system, the well-differentiated H4-II-E rat hepatoma cell line. In H4-II-E cells, IGFBP-1 gene transcription is increased by the synthetic glucocorticoid dexamethasone, and rapidly (<20 min) decreased by physiological concentrations of insulin.

The mammalian IGF-II/Mannose-6-phosphate receptor is a bifunctional receptor that binds IGF-II and proteins containing mannose-6-phosphate recognition markers (such as lysosomal enzymes). Chick embryo fibroblasts contain a primitive form of this receptor. It is the same size as and immunologically related to the mammalian IGF-II/Man 6-P receptor, binds and internalizes lysosomal enzymes, but does not bind IGF-II.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-DK 55007-13 MCNE

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Insulin-cell interaction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I. T.M.Weber	Staff Fellow	MCNEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Molecular, Cellular and Nutritional Endocrinology Branch

SECTION
Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION
NIDDK, NIH,Bethesda, Maryland 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Project inactive.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-DK-55008-13

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S.W. Cushman CHief, EDMNS MCNEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK,NIH,Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Project inactive.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 50009-10 MCNE

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Alterations in insulin's action in insulin-dependent diabetes mellitus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. S.W. Cushman Chief, EDMNS MCNEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK,NIH,Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Project inactive.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 55012-09 MCNE

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Insulin's Regulation of Hormone Binding

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S.W. Cushman Chief, EDMNS MCNEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH,Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project remains current but no summary was available.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 55013-08 MCNE

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Counterregulation of Insulin's Action by Catecholamines

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. A. Simpson Visiting Scientist MCNEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK,NIH,Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 55014-08 MCNE

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Alterations in Insulin's Action with Fasting/Refeeding

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S.W. Cushman	Chief, EDMNS	MCNEB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK,NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project inactive.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK55015-02 MCNE

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mutations of the Thyroid Hormone Receptor Gene in Patients with Thyroid Hormone Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B.D. Weintraub, M.D.	Chief	MCNEB, NIDDK
Others:	A.J. Mixson	Senior Staff Fellow	MCNEB, NIDDK
	P. Hauser	Special Volunteer	MCNEB, NIDDK
	C.A. Meier	Visiting Associate	MCNEB, NIDDK
	S.R. Lasky	Special Expert	MCNEB, NIDDK
	S.C. Ransom	Senior Staff Fellow	MCNEB, NIDDK

COOPERATING UNITS (if any)

Dr. Stephen J. Usala, Section of Endocrinology, East Carolina School of Medicine, Greenville, NC

LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch

SECTION Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 3.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Generalized thyroid hormone resistance is a disorder of thyroid hormone action characterized by elevated free thyroid hormones and TSH, as well as inappropriate clinical and biochemical signs of euthyroidism or hypothyroidism. Using restriction fragment length polymorphism, we initially linked this disorder to one of the two known thyroid hormone receptor genes, c-erbA-beta on chromosome 3. Subsequently we have shown in several families that this disease is caused by mutations in the ligand binding domain of the beta receptor. In each family characterized there have been unique defects all of which have resulted in nonconservative changes in amino acids that have altered the T3 binding properties of the receptor, while causing no apparent change in DNA binding properties. There was a good correlation between decreased T3 binding and decreased transcriptional activity assessed in a cotransfection assay. In addition, all mutations demonstrated a dominant negative effect on the action of normal receptors. These data suggest that decreased binding of T3 prevents a conformational change in the mutant receptor necessary for transcriptional activity.

We are currently elucidating the molecular defects in additional families studied at NIH and are attempting to correlate the unique molecular defects with various clinical manifestations of this syndrome. We are also attempting to express high levels of the receptor using various expression systems. Such studies should allow for the first time an elucidation of the physiologic role of the a and b thyroid hormone receptors for mediating thyroid hormone action in man.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

ANNUAL REPORT OF THE LABORATORY OF STRUCTURAL BIOLOGY
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. The Biology of Complex Carbohydrates

Circumsporozoite (CS) proteins, which densely coat malaria (Plasmodia) sporozoites, contain an amino acid sequence that is homologous to segments in other proteins which bind specifically to sulfated glycoconjugates. The presence of this homology suggests that sporozoites and CS proteins may also bind sulfated glycoconjugates. This hypothesis was tested and results indicate that CS proteins and sporozoites bind specifically to certain sulfated glycoconjugates.

Naturally occurring oligosaccharides, even if isolated from a single protein glycosylation site, can present a daunting array of structures for the glycobiologist to analyze. The molecular details of how glycoconjugate-binding proteins interact with their ligands have been revealed by a variety of techniques.The molecular details of how glycoconjugate-binding proteins interact with their ligands have been revealed by a variety of techniques. For example, proteases, chemical modifying reagents, and antibodies have served as effective probes of lectin functional domains. Highly focused regions of sequence homology has indicated that many structures within the lectin glycoconjugate-binding domains themselves may be conserved.

Mannose-binding protein (MBP)[1] binds with high affinity to glycoconjugates containing terminal mannose or N-acetylglucosamine residues, and binds more weakly to those containing terminal fucose residues. Mannose-binding protein was purified from human serum to apparent homogeneity by affinity chromatography on mannose-Sepharose, followed by affinity chromatography on underivatized Sepharose. Approximately 0.4 mg protein was obtained from 1 liter serum. The glycosphingolipid binding specificity of the purified protein was examined by chromatogram overlay and solid phase assays. The binding specificity was described.

II. Expression and Function of Bacterial Cell Surface Components in Pathogenesis

A bacterial pathogen must be able to invade and subsequently multiply, or at least survive, in its host. Normally, the host defenses, such as serum-killing and phagocytosis, successfully oppose the invader and where an infection becomes established, administration of antibiotics may provide additional time for the host to eliminate invading pathogen. These studies focus on the how the cell surface components of pathogenic bacteria function in resistance to host defense mechanisms and antibiotics.

A. Escherichia coli, Salmonella typhimurium, and other Gram-negative bacteria become phenotypically resistant to a variety of unrelated antibiotics when grown in media containing

0.5-10 mM acetaminophen. The increased antibiotic resistance is due, at least in part, to the decreased expression of porin protein involved in the permeation of the various antibiotics through the outer membrane barrier. This effect is similar, if not identical, to that seen when these same cells are grown in sodium salicylate or acetylsalicylate.

B. 56 Gram-negative strains, isolated as human pathogens, were grown in the presence of sodium salicylate and their sensitivity to each of four antibiotics tested [Cefoxitin, Ciprofloxacin, Norfloxacin, and Tetracycline]. All strains showed a minimally 4 fold increase in the minimal inhibitory concentration [MIC] of at least one antibiotic and most showed significant increase in the MIC of all 4 antibiotics studied. The 56 strains studied included species of Acinetobacter, Citrobacter, Enterobacter, Escherichia, Klebsiella, Proteus.

C. Several genetic loci are involved in the regulation of the expression of the Escherichia coli outer membrane porins. These loci include ompR, envZ, and micF. The relationship of these loci with the observed regulation of expression of Omp F porin in the outer membrane, various lacZ$^{\pm}$ fusion strains were grown in the presence or absence of added salicylate. The effect of salicylate on ompF expression was independent of the osmolarity of the medium. It was epistatic to the envZ3 and ompR331 osmoregulatory mutations. Like salicylate, these mutations, engender high constitutive levels of micF transcription and concomitant low levels of OmpF porin expression. These results showed that growth in salicylate severely decreases the translation of ompF by enhancing the expression of micF. In this respect, sal/-grown cells resemble marA or tolC mutant strains that have high levels of micF and low levels of OmpF.

III. Metabolism and role of Polysaccharide Sulfates

This project has been terminated. Dr. Leader has continued these studies, working on a volunteer basis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-57000-26 LSB

PERIOD COVERED
October 1, 1990 - September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Biology of Complex Carbohydrates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Victor Ginsburg	Lab Chief	LSB, NIDDK
Others:	Gordon Holt	Senior Staff Fellow	LSB, NIDDK

COOPERATING UNITS *(if any)*
James Mulshine, NCI, NIH

LAB/BRANCH
Laboratory of Structural Biology

SECTION
Biochemistry

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Circumsporozoite (CS) proteins, which densely coat malaria (Plasmodia) sporozoites, contain an amino acid sequence that is homologous to segments in other proteins which bind specifically to sulfated glycoconjugates. The presence of this homology suggests that sporozoites and CS proteins may also bind sulfated glycoconjugates. This hypothesis was tested and results indicate that CS proteins and sporozoites bind specifically to certain sulfated glycoconjugates.

Naturally occurring oligosaccharides, even if isolated from a single protein glycosylation site, can present a daunting array of structures for the glycobiologist to analyze. The molecular details of how glycoconjugate-binding proteins interact with their ligands have been revealed by a variety of techniques.The molecular details of how glycoconjugate-binding proteins interact with their ligands have been revealed by a variety of techniques. For example, proteases, chemical modifying reagents, and antibodies have served as effective probes of lectin functional domains. Highly focused regions of sequence homology has indicated that many structures within the lectin glycoconjugate-binding domains themselves may be conserved.

Mannose-binding protein (MBP)1 binds with high affinity to glycoconjugates containing terminal mannose or N-acetylglucosamine residues, and binds more weakly to those containing terminal fucose residues Mannose-binding protein was purified from human serum to apparent homogeneity by affinity chromatography on mannose-Sepharose, followed by affinity chromatography on underivatized Sepharose. Approximately 0.4 mg protein was obtained from 1 liter serum. The glycosphingolipid binding specificity of the purified protein was examined by chromatogram overlay and solid phase assays. The binding specificity was described.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 57001-14 LSB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Metabolism and Role of Polysaccharide Sulfates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Irwin G. Leder	Research Chemist	LSB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Structural Biology

SECTION
Section on Biochemistry

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-57002-17 LSB

PERIOD COVERED
October 1, 1990 - September 39, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Expression & Function of Bacterial Cell Surface Components in Pathogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: John Foulds Research Biochemist LSB, NIDDK

COOPERATING UNITS (if any)

Judeh Rosner LBM, NIDDK

LAB/BRANCH
Laboratory of Structural Biology

SECTION
Micribial Membranes

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A bacterial pathogen must be able to invade and multiply in its host. Host defenses, such as serum-killing and phagocytosis, successfully oppose the invader and where an infection becomes established, administration of antibiotics may provide additional time for the host to eliminate invading pathogen. These studies focus on the how the cell surface components of pathogenic bacteria function in resistance to host defense mechanisms and antibiotics.

Escherichia coli, Salmonella typhimurium, and other Gram-negative bacteria become phenotypically resistant to a variety of unrelated antibiotics when grown in media containing 0.5-10 mM acetaminophen. The increased antibiotic resistance is due to the decreased expression of porin protein. This effect is similar, if not identical, to that seen when these same cells are grown in sodium salicylate.

56 Gram-negative strains, isolated as human pathogens, were grown in the presence of sodium salicylate and their sensitivity to each of four antibiotics tested [Cefoxitin, Ciprofloxacin, Norfloxacin, and Tetracycline]. All strains showed a minimally 4-fold increase in the minimal inhibitory concentration [MIC] of at least one antibiotic and most showed significant increase in the MIC of all four antibiotics studied. The 56 strains studied included species of Acinetobacter, Citrobacter, Enterobacter, Escherichia, Klebsiella, Proteus.

Several genetic loci are involved in the regulation of the expression of the Escherichia coli outer membrane porins. The regulation of expression of Omp F in the outer membrane, various lacZ+ fusion strains were grown in the presence or absence of salicylate. The effect of salicylate on ompF expression was independent of the osmolarity of the medium. It was epistatic to the envZ3 and ompR331 osmoregulatory mutations.

Like salicylate, these mutations, engender high constitutive levels of micF transcription and concomitant low levels of OmpF porin expression. These results showed that growth in salicylate severely decreases the translation of ompF by enhancing the expression of micF. In this respect, sal-grown cells resemble marA or tolC mutant strains that have high levels of micF and low levels of OmpF.

Dr. Foulds was detailed to the National Vaccine Program Office for part of the year [March 15-September 30] where he served as a policy analyst and scientific advisor to the program.

ANNUAL REPORT

THE LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The LMCB comprises several diverse groups studying a common theme of gene regulation in mammalian cells and their function in the pathophysiology of various disease states. The Molecular Biology Section led by Dr. Carter, studies gene regulation in mammalian cell systems and is particularly interested in developing efficient vector systems for delivery of genes into cells. The Cell Growth and Differentiation Section led by Dr. Oka, is generally interested in the endocrine control of differentiation of the mouse mammary gland and has focused on physiological effects of EGF and the molecular biology of various genes which are important in this process. The Steroid Hormones Section led by S. Simons studies the mechanism of regulation of genes via steroid hormones and steroid receptors using techniques of chemistry and molecular biology. Finally a fourth project led by Dr. Tietze and conducted in collaboration with workers in NICHD is aimed at understanding the molecular basis of several human genetic defects which result in lysosomal storage diseases.

Research performed in LMCB has been recognized by various honors, awards and grants to individual members. B. Carter has a substantial three year research grant from the Cystic Fibrosis Foundation for gene therapy of cystic fibrosis. T. Flotte was awarded a Leroy Matthews Physician Scientist Award from the Cystic Fibrosis Foundation to work in the Molecular Biology Section. C. Collier was awarded a PRAT Fellowship to work in the Steroid Hormones Section.

B. Carter was a speaker at the Regional Hearings of the NIH DNA Recombinant Advisory Committee (October 1990); an Invited Symposium Speaker at the International Cystic Fibrosis Meeting (Arlington VA October 1990); an Invited Speaker at the 1991 Williamsburg Conference on Cystic Fibrosis (Williamsburg, VA June 1991); will be an Invited Symposium Speaker at the 5th Annual North American Cystic Fibrosis Conference (Dallas, TX October 1991) and will chair a Workshop Session on Gene Therapy at the same meeting. B. Carter will also be a Session Chairman at the NIH/NHLBI Meeting on Gene Therapy (Bethesda, MD December 1991).

T. Flotte was also an Invited Speaker at the International Cystic Fibrosis Meeting (1990), the Williamsburg Cystic Fibrosis Conference (1991) and the Fifth Annual North American Cystic Fibrosis Conference (1991).

S. Simons is an Invited Speaker at the Endocrine Society Annual Meeting June 1991 (Washington, DC) and the 4th International Congress on Hormones in Cancer, September 1991 (Amsterdam, Holland).

T. Oka was an Invited Speaker at the International Congress on Polyamines, November 1990 (Japan); The Biennial Meeting of the International Association for Breast Cancer Research, May 1991 (Italy); The International Symposium on Biotechnology and Animal Production April 1991 (Chile); and the FASEB Annual Meeting, April 1991 (Atlanta, GA).

F. Tietze was an Invited Speaker in the Plenary Symposium of the 5th International Congress on Inborn Errors in Metabolism, June 1990 (Asilomar, CA).

B. Carter continues to serve fourth terms as an Associate Editor of Virology and on the Editorial Board of Journal of Virology and continues to serve on the WHO International Committee for Nomenclature of Viruses, as

Chairman of the NIH Institutional BioSafety Committee and as a member of the NIH Intramural Scientists Procurement Advisory Committee.

S. Simons continues to serve on the Editorial Board of Receptors and has been appointed to the Editorial Board of Journal of Biological Chemistry. S. Simons was also elected President of the NIDDK Assembly of Scientists.

Several members of the laboratory including T. Oka, F. Tietze and S. Simons were invited to write a number of critical reviews in their fields.

Members of the laboratory have maintained international collaborations with groups in Tokyo (Japan), Bet-Dagan (Israel), Tel Aviv (Israel) and Basle (Switzerland) as well as collaborations with U.S. laboratories at Rutgers (N.J.), Toledo (Ohio), Ann Arbor (Michigan), U. California (San Francisco) and John's Hopkins Medical School (Baltimore).

Function of DNA Virus Genomes in Animal Cells

The group led by B. Carter has continued to employ DNA viruses as molecular probes to study genome expression in human cells. The structure and function of adeno-associated virus (AAV) is being studied intensively. AAV has also been developed as a eukaryotic expression vector.

In a newly developed project, sponsored in part by the Cystic Fibrosis Foundation, we are attempting to develop vectors for possible gene therapy of cystic fibrosis (CF). Thus far the CF defect has been complemented in airway epithelial cells in vitro by introduction of an AAV vector containing the cDNA for the CFTR gene. These complementation studies have been achieved both by introducing the AAV vector DNA via cationic liposomes or by infection with AAV transducing particles (i.e. AAV particles containing the vector genome). The latter procedure is remarkably efficient and can provide stable correction of the CF defect in the bulk of the cells in the population. These studies show that the AAV vector is a strong possibility for a gene therapy for CF. Animal studies with AAV vectors are now being initiated prior to proposing human gene therapy trials.

A complex system of gene regulation is mediated by products of the AAV rep gene is are required for replication of AAV DNA but also mediate transcriptional activation and translational inhibition of some genes. Site-directed mutagenesis is being used to resolve the functions of the rep gene. Coding of all these functions in a single gene appears to be unique in eukaryotic systems. A number of very informative mutants of the AAV rep gene were generated. Two alternate expression systems were developed (one using baculovirus vectors in insect cells and one using a transfection system in human cultured cells) which allows expression of mutant AAV rep proteins at high efficiency. This has been used to show that one particular mutant rep protein (which has a DNA-negative, trans-dominant phenotype in vivo) is also trans-dominant in vitro. In vitro the mutant rep protein can bind to the AAV replication origin but cannot perform terminal resolution (the first step in AAV DNA replication). Further the mutant rep protein blocks in vitro terminal resolution by the wild type rep protein.

A third project is analysing the interaction between AAV and HIV. Current work suggests that production of vaccines or use of nucleotide analog therapies for AIDS may be limited and difficult. Thus other therapeutic approaches are urgently required. Attempts are underway to develop a novel approach by using the negative regulatory property of a trans-acting gene of the human parvovirus, AAV, to inhibit the function of the trans-acting gene tat, of Human Immunodeficiency Virus (HIV). We have shown in transfection assays that the AAV rep protein can be engineered to be a powerful inhibitor of growth of infectious HIV. We are now analysing the precise mechanism of this effect and attempting to develop delivery systems to introduce Rep into HIV infected cells such that it would be used as an anti-viral therapy for AIDS.

Hormonal Regulation of Cell Growth and Differentiation.

Dr. Oka's group in the Section of Cell Growth and Differentiation is studying the mouse mammary gland and also the function of EGF.

Epidermal growth factor (EGF) is produced in large amounts by the mouse submandibular gland, is a potent mitogen for a wide variety of cells in culture but its function in the body needs to be elucidated. Dr. Oka's studies have demonstrated that EGF plays a key role in the development of the mammary gland during pregnancy and mammary tumorigenesis in female mice; in males it serves a role in spermatogenesis by stimulating the meiosis of spermatocytes. Studies have been continued to elucidate the physiological role of EGF by employing a variety of experimental approaches.

The role of EGF in liver regeneration, was examined in normal and sialoadenectomized mice after partial hepatectomy. Several lines of evidence indicate that endogenous EGF secreted from the submandibular glands promotes liver regeneration.

A model system in which direct effects of androgens on the liver can be evaluated in vivo has been developed. The system employed intrasplenic implantation of a proper dose of a testosterone pellet, so that testosterone reaches the liver through the portal vein without entering the systemic circulation due to rapid clearance by the liver. By use of this system, it has been shown that testosterone directly enhanced hepatic levels of EGF receptor MRNA and EGF binding.

Molecular biology studies are being used to analyse the synergistic actions of the hormones insulin, glucocorticoid and prolactin to regulate casein expression in the mouse mammary gland. Genomic clones of the mouse casein gene were isolated and sequenced. Chimeric genes containing a reporter CAT gene fused to the casein promoter region were transfected into mammary epithelial cells and showed appropriate hormone responses. Additional studies identified both positive and negative control elements.

The chimeric casein-CAT gene was transfected into primary mouse mammary epithelial cells prepared from pregnant mice. Expression of the chimeric gene required the synergistic actions insulin, hydrocortisone, and prolactin and also depended on the appropriate (i.e. reconstituted) basement membrane (Matrigel). Additional transfection experiments with a series of β-casein-CAT constructs suggested the existence of regulatory elements in the 500 bp 5'-flanking region which is responsible for hormonal induction.

Steroid Hormone Action

The action of glucocorticoid hormones is being studied by Simons' group in the Steroid Hormones Section. Steroid-induced gene transcription occurs after the receptor-steroid complex binds to specific DNA sequences. A poorly understood step is that of steroid binding to the receptor. Several approaches combining biochemistry, molecular biology, organic chemistry, and immunochemistry are being used to study this. With several methods there was no evidence for glycosylation of the rat glucocorticoid receptor. In contrast association with heat shock protein 90 (hsp90) is required. To identify the hsp90 binding site in the receptor, evidence of hsp90 association with the 16 kDa steroid binding fragment of the rat glucocorticoid receptor (i.e., Thr-537 to Arg-673) has now been obtained. The 16 kDa fragment was also immunoprecipitable with anti-hsp90 antibody. These results argue that hsp90 binds to the 16 kDa core fragment.

Affinity labeling is a very powerful method for identifying amino acids in ligand binding cavities. Dexamethasone 21-mesylate (Dex-Mes) that affinity labels only one amino acid of the rat glucocorticoid receptor, (Cys-656) was previously developed. A new affinity label for glucocorticoid receptors, dexamethasone 21-thio (β-isothiocyanato)ethyl either (Dex-NCS) was developed.

Data on the reactivity of Dex-NCS and the stability of [^{3}H]Dex-NCS-labeled receptors suggest that a cysteine-SH group was labeled.

Previous experiments with the thiol reagents methyl methanethiolsulfonate (MMTS) and sodium arsenite implicated the existence of a vicinal dithiol group in the steroid binding cavity. Additional results suggested that the vicinal dithiols were two of the three cysteines (Cys-640, -656, and -661 of the rat receptor) found in the 16 kDa trypsin fragment of the receptor. The steroid binding specificity of the 16 kDa fragment is virtually identical to that of the intact 98 kDa receptor. This finding establishes the 16 kDa fragment as the steroid binding core element. Mutagenesis showed that no one cysteine was required for steroid binding but only those mutants containing an intact Cys-656 were covalently labeled by [^{3}H]Dex-Mex. These data support a model for the steroid binding cavity that consists of a flexible cleft.

Almost all modifications of the steroid-binding domain of glucocorticoid receptors are known to cause a reduction or loss of steroid binding activity. However, mutations of cysteine-656, to give Cys656Ser and Cys656Gly produced "super" receptors, with a 3-9 fold increased affinity for glucocorticoid steroids. Additional modifications in the steroid binding domain of glucocorticoid receptors, and probably other steroid receptors, may further increase the binding affinity and/or specificity.

Lysosomal Transport and Storage Disease

This work is being conducted by Dr. Frank Tietze. Degradation of cellular biopolymers such as proteins and polysaccharides takes place chiefly within the lysosome. The end-products of lysosomal digestion of biopolymers are known to exit the lysosome through the agency of specific membrane carrier proteins. Defects in these carrier proteins can result in a number of inherited storage disorders which are characterized by intralysosomal entrapment of excessive amounts of specific metabolites such as cystine or sialic acid. Unlike the plasma membrane carrier proteins, many of which have been isolated and/or sequenced, nothing is known concerning the structure of the lysosomal membrane carriers. Lack of an appropriate in vitro assay for carrier function has served as a serious obstacle to such attempts. We are currently investigating such an assay system through the use of reconstituted artificial lipid-membrane vesicle preparations (proteoliposomes). Uptake of specific radio-labeled ligands such as cystine or sialic acid would presumably signify retention of functional carrier activity by the lysosomal membrane fractions and assist in monitoring their further purification.

Preliminary attempts have been made to reconstitute integral lysosomal membrane proteins into artificial lipid vesicles to form proteoliposomes possessing specific carrier functions of the native, intact lysosome. Reconstituted proteoliposomes from rat liver lysosomal membranes have been prepared and their ability to incorporate radiolabeled cystine or sialic acid examined. To date it has been difficult to demonstrate significant uptake of either of these ligands with our vesicle preparations. Part of this difficulty is due to the extremely small internal volume of the vesicles and this limitation is being overcome by procedures known to produce larger ("giant") vesicles such as freezing in liquid nitrogen. Attempts are also being made to increase the magnitude of uptake beyond equilibrium levels by transstimulation effects, i.e., by incorporating high concentrations of non-radioactive ligand within the vesicles at the time of their formation during detergent removal.

Summary

Research in LMCB overall has followed a common theme of performing basic science on gene expression and also applying this to study of disease states. Thus recent work from this laboratory has identified the basic biochemical defect in one genetic disease (sialuria) illuminated a possible role of EGF deficiency in another disease (diabetes), and is aimed at developing therapeutic approaches to an infectious disease (AIDS) and a genetic disease (cystic fibrosis).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57501-15

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Function of DNA Virus Genomes in Animal Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Barrie J. Carter	Chief, LMCB	LMCB:NIDDK
Other:	Irving Miller	Biologist	LMCB:NIDDK
	Roland Owens	IRTA Fellow	LMCB:NIDDK
	Matthew Weitzman	Visiting Fellow	LMCB:NIDDK
	Terry Flotte	NIH-Johns Hopkins CF Fellow	LMCB:NIDDK
	Rikki Solow	Guest Worker	LMCB:NIDDK
	Sandra Afione	Guest Worker	LMCB:NIDDK
	Sirkka Kyostio	Visiting Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*

J.P. Trempe (Toledo, OH), N. Chejanovsky (Israel), P. Zeitlin (John's Hopkins)
D.F. Klessig (Rutgers), E. Mendelson (Tel Aviv, Israel), M. Drumm (Michigan)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of a human parvovirus, adeno-associated virus (AAV). AAV has been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes into mammalian cell chromosomes to yield stable expression. This vector also may be useful for gene therapy. We are now analyzing intensely the control of gene regulation in AAV vectors in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. We are developing AAV vectors that express the CFTR gene as a potential gene therapy for cystic fibrosis. We have discovered a complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediate transcriptional activation and translational inhibition of some genes. Site-specific mutagenesis is being used to resolve these functions. Coding of all these functions in a single gene is unique in eukaryotic systems. Adenovirus is the helper for AAV. This relationship is being analyzed. Both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus, AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in cell culture. We also are analyzing interactions of AAV with HIV as a potential approach to a novel therapy for AIDS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57502-18

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hormonal Regulation of Cell Growth and Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Takami Oka	Senior Investigator	LMCB:NIDDK
Other:	Akio Kanai	Visiting Fellow	LMCB:NIDDK
	Chang-Soo Lee	Visiting Fellow	LMCB:NIDDK
	Robert Moore	IRTA Fellow	LMCB:NIDDK
	Shoji Oda	Guest Worker	LMCB:NIDDK
	Yoshito Ohba	Visiting Fellow	LMCB:NIDDK
	John W. Perry	Biologist (Technician)	LMCB:NIDDK

COOPERATING UNITS *(if any)*

Dr. Kishio Furuya, National Institute of Physiological Sciences, Japan
Dr. Koichi Enomoto, Shimane Medical University, Japan

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Cell Growth and Differentiation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epidermal growth factor (EGF) is a single-chain peptide (mol wt 6,045) that was originally isolated from the submandibular glands of male mice. EGF enhances the proliferation of various mammalian cells in culture but its physiological role remains to be clarified. In mice the submandibular gland is the major site of EGF production and the major source of circulating EGF. Previous studies employing experimentally induced (sialoadenectomized) EGF-deficient mice have suggested that EGF plays an important role in many physiological processes including spermatogenesis, completion of normal pregnancy and wound healing. Thus the possibility exists that EGF deficiency contributes to the pathology of disease states. Diabetes mellitus is of interest in this respect, since EGF deficiency occurs in diabetes mellitus and some of the pathology accompanying the disease includes disorders ordinarily associated with EGF deficiency. Oligozoospermia is one of the pathological complications associated with diabetes mellitus, but its etiology is not fully understood. We have shown that EGF is important for maintaining normal sperm production in mice. Removal of the submandibular gland results in the reduced level circulating EGF and about a 50% loss of mature sperm in the epididymis, whereas supplementation with EGF can fully restore the sperm counts to normal levels. Our studies have shown that in two kinds of diabetic mice, namely genetically diabetic mice and mice with streptozotocin (STZ) induced diabetes the levels of prepro-EGF-mRNA and EGF in both the submandibular gland and plasma are greatly reduced. No apparent difference in the level of kidney prepro-EGF mRNA was found between the diabetic and control mice. The epididymal sperm counts of diabetic mice were significantly lower than those of normal mice. Administration of EGF to STZ-induced diabetic mice substantially increased their sperm counts, whereas insulin treatment of diabetic mice restored the submandibular and plasma EGF concentrations and sperm counts to normal levels. The restorative effects of insulin on sperm production appeared to be mediated at least in part by EGF because of its effect was blocked by the concomitant administration of EGF antiserum. These results suggest that EGF deficiency is a possible cause for the pathogenesis of oligozoospermia in diabetic mice.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57503-18

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Metabolic Defect in Sialuria

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Frank Tietze Research Chemist LMCB:NIDDK

Other: None

COOPERATING UNITS (if any)

William A. Gahl, Human Genetics Branch, NICHD

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The end-products of lysosomal digestion of biopolymers (amino acids, monosaccarides, nucleosides, etc.) are known to exit the lysosome through the agency of specific membrane carrier proteins. It is currently believed that in humans, defects in these carrier proteins can result in a number of inherited storage disorders which are characterized by intralysosomal entrapment of excessive amounts of specific metabolites such as cystine or sialic acid. Unlike the plasma membrane carrier proteins, many of which have been isolated and/or sequenced, nothing is known concerning the structure of the lysosomal membrane carriers. Among the approaches which may be followed to accomplish this characterization is their purification from isolated lysosomal membrane vesicles. However, lack of an appropriate *in vitro* assay for carrier function has served as a serious obstacle to such attempts. We are currently investigating such an assay system through the use of reconstituted artificial lipid membrane vesicle preparations (proteoliposomes). Uptake of specific radio-labeled ligands such as cystine or sialic acid would presumably signify retention of functional carrier activity by the lysosomal membrane fractions and assist in monitoring their further purification.

PHS 6040 (Rev. 1/84) GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57504-04

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of HIV by AAV

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Barrie J. Carter	Chief, LMCB	LMCB:NIDDK
Other:	Roland Owens	IRTA Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*

A.S. Rabson and B. Antoni (Rutgers, NJ)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The etiologic agent of AIDS is the human immunodeficiency virus (HIV) which differs from most other human viral diseases in exhibiting a very prolonged latent period, but ultimately being lethal due to a profound effect on the immune system. Several trans-acting HIV genes appear to be crucial to HIV growth and infection. Therefore we are studying the feasibility of a novel anti-viral therapy for HIV based on interference by another viral gene with the trans-acting regulation of HIV. The overall goal of this proposal is to analyze interactions between trans-acting regulatory genes of HIV and of a human parvovirus, adeno-associated virus, AAV. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. Current work suggests that developing standard types of anti-viral therapy such as vaccines or nucleotide-analog drugs for HIV is difficult and other alternate possibilities for therapy must be investigated. One approach is to intervene in the trans-regulation system of HIV especially that mediated by the HIV tat gene. Thus a possible anti-viral therapy for HIV is to inhibit the production or the action of tat. A novel way to attempt this is to employ a trans-acting gene from another human virus. One such candidate is the rep gene of the human parvovirus adeno-associated virus (AAV). AAV does not cause any human disease and grows only in cells also infected with adenovirus or herpes viruses. AAV inhibits growth of the helper virus and may play an important role in limiting certain human viral infections. Also AAV can alter important regulatory controls in virus infected cells or in tumor cells. Rep is a novel type of trans-acting regulatory gene which exhibits negative, translational regulation of many genes in several cell types. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. We are testing rep as a potential trans-acting heterologous inhibitor of HIV.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58002-16

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Initial Intracellular Events of Steroid Hormone Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	S.S. Simons, Jr.	Chief, Steroid Hormones Section	LMCB:NIDDK
Other:	A.H. Cavanaugh	Staff Fellow	LMCB:NIDDK
	H. Oshima	Visiting Fellow	LMCB:NIDDK
	P.K. Chakraborti	Visiting Associate	LMCB:NIDDK
	Y. Miyashita	Visiting Fellow	LMCB:NIDDK
	D. Szapary	Visiting Associate	LMCB:NIDDK
	J. Opoku	IRTA Fellow	LMCB:NIDDK
	C.D. Collier	IRTA Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*

W.B. Pratt (Univ. of Michigan Medical School, Ann Arbor),
K.R. Yamamato (UCSF, San Francisco)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Steroid Hormones Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 8.7	PROFESSIONAL: 7.7	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to define the initial, intracellular events of glucocorticoid hormone action, and steroid hormone action in general. The first step of steroid binding to the intracellular receptor molecule is followed by activation of the receptor-steroid complex to a DNA-binding and nuclear-binding species and then binding of activated complexes to those nuclear acceptor sites involved in the regulation of transcription of specific genes. A combination of techniques have been used to examine the crucial first step for glucocorticoid steroids. Biochemistry, affinity labeling, and proteolysis have been used to define a steroid binding core element of the receptor which contains most, if not all, of the amino acids required for specific steroid binding along with the ability to bind heat shock protein 90. Metabolic labeling and immunochemistry revealed that posttranslational glycosylation does not appear to be required for the steroid binding activity of the receptor. Molecular biology, used to create point mutations of the 3 cysteines in the steroid binding core element, has yielded "super" receptors with increased binding affinity and specificity for glucocorticoids. Such "super" receptor may prove to be of use in future genetic engineering treatment of patients with defective receptors and/or steroid biosynthesis. Finally, a rational examination of the steroid binding domains of all the steroid receptors for the presence of potential vicinal dithiols permitted us to devise a protocol involving pretreatment with sodium arsenite that specifically inactivates only glucocorticoid receptors. This procedure should be of great assistance in the quantitation of progesterone and mineralocorticoid receptors. Future studies concerning the molecular details of steroid-receptor interactions should provide further information which will aid in understanding the control of steroid-regulated gene transcription and the treatment of patients.

ANNUAL REPORT OF THE
LABORATORY OF ANALYTICAL CHEMISTRY
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Following a reorganization last year of several Laboratories oriented around various aspects of organic chemistry, the primary mission of the Laboratory of Analytical Chemistry (LAC) now focuses on the provision of analytical services for investigators in NIDDK and elsewhere at NIH and the development of improved analytical methods. Many of the analytical problems brought to LAC for solution involve only application of relatively routine methods, while others are of such complexity that collaborative research efforts are needed.

Included in this report for administrative convenience is the report of the Natural Products Section, even though it is formally no longer a part of LAC. This section will be abolished at the end of this fiscal year when its Chief, Dr. Arnold Brossi, retires after a distinguished research career, much of it in the pharmaceutical industry, but the last 15 years in NIDDK.

INSTRUMENTATION SECTION

During the last year three new instruments have been put into service to augment the Laboratory's capabilities in nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared (IR) spectroscopy. A Varian Gemini 300 MHz NMR instrument is now available for "hands on" operation by NIH investigators. Its sophisticated software permits fully automatic operation for routine samples, while providing simple, menu-driven procedures for more complex one dimensional and two dimensional studies. A Digilab FTS-40 Fourier transform IR spectrometer provides modern capability to analyze solid and liquid samples. A JEOL SX-102 high resolution mass spectrometer, equipped for fast atom bombardment, provides enhanced capabilities for handling non-volatile samples and permits more accurate mass measurements than available in LAC heretofore. In addition, LAC instrumentation is augmented by the availability of a californium plasma and UV matrix assisted desorption mass spectrometer and ion trap mass spectrometer in the Laboratory of Biophysical Chemistry (LBC), NHLBI.

Developments in Mass Spectrometry

During the last year it has become apparent that normally non-volatile molecules of high molecular weight, such as proteins, can be studied by MS using recent developments in laser desorption and electrospray technology. During this year, the potential mass range for routine samples analyzed within the laboratory has risen from 1,000 to 50,000 with the introduction of these new techniques and instrumentation, In collaboration with LBC, NHLBI, an inter-Institute macromolecular structure determination center is being established. Further information on MS studies and collaborative research problems is included in the annual report of the Laboratory of Bioorganic Chemistry, NIDDK. (Lewis Pannell)

Applications of NMR in Biochemical and Biological Systems (H.J.C.Yeh)

Advances in sophisticated pulse sequences (eg., 2D Relayed COSY, HMQC etc.) and the availability of a high field magnet have made structural assignment and conformational study of molecules with submilligram quantities possible. Using these methods on our 500 MHz NMR spectrometer, a number of compounds provided by other researchers have been investigated and their structures have been successfully determined. This includes 1) submilligram quantity of epibatidine, a novel chloropyridyl azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog. (T. Spande and J. Daly); 2) new ketodienes from culticular lipids of the Guam brown tree snake (R. Mason, Y. Murata, L. Pannell, T. Jones and H. Fales); 3) microbial oxidative metabolites from the action of enzymes in Beauveria sulfurescens on the antimalarial drug arteether (Y.L. Hu, H. Ziffer and G.Y. Li); 4) polysaccharides (P. Kovac), 5) colchinoids (0. Boye and A. Brossi); 6) covalent nucleoside adducts of diol epoxides derived from polycyclic aromatic hydrocarbons (J. Sayer and D. M. Jerina); 7) oxahalide complexes of molybdenum and tungstem with oxygen donor ligands (F. Planinic and D. Vikic-Topic); 8) metabolites from the action of purine nucleoside phosphorylase on purine analogs.

In collaboration with Dr. E. Moody and Dr. P. Skolnick, ^{19}F NMR spin-spin relaxation time (T_2) has been measured on halothane in brain and other tissues of rats. We find that the highly immobilized environment for halothane, characterized by the short T_2, as reported earlier by another group is not unique to brain. Short T_2 environments for halothane were also obtained in packed red blood cells and other peripheral tissues of anesthetized rats. While the presence of the short T_2 for halothane in brain (and other tissues) may be of interest, our findings indicate it is not directly related to the molecular mechanisms of anesthesia.

Purine nucleoside phosphorylases (PNP) are enzymes that catalyze the cleavage of the glycosidic bond of purine nucleosides. The reaction is reversible and indeed favors the formation of nucleosides. Using NMR, which permits monitoring of all reaction products simultaneously, we are currently investigating the geospecificity and mechanism of the formation of nucleoside from the corresponding bases and sugar-1-phosphates in the presence of this enzyme (G.Y. Li).

NATURAL PRODUCTS SECTION

In attempts to recognize and fully characterize the colchicine receptor on tubulin several isothiocyanates of thiocolchicine and allo-colchicine congeners were prepared. It is expected that 9-isothiocyanato-9-deoxy-N-acetylcolchinol, the most interesting of them, will undergo specific and covalent interaction. For this investigation the isocyanate will be prepared with a radiolabel in the methoxy group at C(2). The synthesis of(-)-physovenine from (-)-eseroline now allows to prepare analogs of the alkaloid with different carbamate groups.

Several have already been made and they were found to be equally potent inhibitors of acetylcholinesterase and of butyrylcholinesterase as the corresponding analogs in the physostigmine series. The chemistry developed also allows to substitute the oxygen atom in the physovenines with a sulfur atom. These thiaphysovenines belong to the most potent inhibitors of cholinesterases ever found. Enzyme specificity has been encountered in analogs of the phenylcarbamate of (-)-eseroline, when the phenyl group was substituted with alkyl groups. One of them is a potent inhibitor of AChE, whereas the other is highly specific for the inhibition of BChE.

Procedures have been developed to prepare from commercially available (±)-6-acetoxytropin-3-one nor-analogs in racemic, and in optically active form. It also is possible to connect these ketones with the corresponding nortropanes by removing the carbonyl group by Raney-Ni desulfurization of dithioketals. A possible biosynthesis of mammalian morphine following a pathway outlined for the morphine biosynthes is in Papaver somniferum differs significantly at the stage of the isoquinoline precursor (S)-3'-hydroxy-N-methylcoclaurine. In plants this isoquinoline is specifically and efficiently converted into (S)-reticuline, whereas with mammalian enzymes a similar efficient conversion afforded (S)-orientaline.

The dihydrofluoresceins DADF and DBDF emerged as useful analytical reagents to characterize amines and alcohols. It has now been shown that it is feasible to prepare optically active dihydrofluoresceins when the two aromatic rings of the xanthene moiety are differently substituted. It is expected that such reagents may be useful for specific staining of tissues.

NMR Research and Service: The NMR laboratory is equipped with two 300 MHz and one 500 MHz instruments. One of the 300 MHz spectrometers, equipped with an automatic sample changer, has been used to handle routine samples. A second 300 MHz (recently installed), which is a hands-on instrument, is available to the researchers in building 8. Approximately 3,500 spectra were obtained during the year, about 75% of which were proton NMR. The 500 MHz spectrometer is used for specialized studies, including most two dimensional NMR methods.

Several projects that involve Dr. Yeh's work in developing specialized techniques and/or in collaborative research are worth highlighting. NMR structural and conformational study of molecules with submilligram quantity and/or with congested resonance peaks often require sophisticated experiments (such as 2D Relayed COSY, HMQC etc.) carried out at the highest possible magnetic field strength for gaining sensitivity and resolution. Using these methods on our high field NMR spectrometer (Varian 500), a number of compounds provided by other investigators have been investigated and their structures have been determined. This includes 1) submilligram quantity of epibatidine, a novel chloropyridyl azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog (T. Spande and J. Daly), 2) new ketodienes from culticular lipids of the Guam brown tree snake (R. Mason, Y. Murata, L.Pannell, T. Jones and H. Fales), 3) microbial oxidative metabolites from the action of enzymes in Beauveria sulfurescens on the antimalarial drug arteether (Y.L. Hu H. Ziffer and G.Y. Li), 4) polysaccharides (P. Kovac), 5) colchinoids (0. Boye and A. Brossi), 6) covalent nucleoside adducts of diol epoxides derived from polycyclic aromatic hydrocarbons (J. Sayer and D. M. Jerina), 7) oxahalide complexes of molybdenum and tungsten with oxygen donor ligands (F. Planinic and D. Vikic-Topic), and 8) metabolites from the action of purine nucleoside phosphorylase on purine analogs.

In collaboration with Dr. E. Moody and Dr. P. Skolnick, ^{19}F NMR spin-spin relaxation time (T_2) has been measured on halothane in brain and other tissues of rats. We find that the highly immobilized environment for halothane, characterized by the short T_2, as reported earlier by other groups is not unique to brain. Short T_2 environments for halothane were also obtained in packed red blood cells and other peripheral tissues of anesthetized rats. While the presence of the short T^2 for halothane in brain (and other tissues) may be of interest, our findings indicate it is not directly related to the molecular mechanisms of anesthesia.

Purine nucleoside phosphorylases (PNP) are enzymes that catalyze the cleavage of glycosidic bond of purine nucleosides. The reaction is reversible and indeed favors the formation of nucleosides. Using NMR, which permits monitoring of all reaction products simultaneously, we are currently investigating the geospecificity and mechanism of the formation of nucleoside from the corresponding bases and sugar-1-phosphates in the presence of this enzyme (G.Y.Li).

MS Research and Service: One MS laboratory, under Mr. Whittaker and Mr. White, is the principal resource for routine MS measurements. During the last year almost 4000 mass spectra were obtained, with a variety of instruments and techniques.

A new JEOL SX102 high resolution mass spectrometer (shared purchase with LBC, NHLBI) has been installed and come into considerable use this year with about 1600 samples being analyzed in 12 months in Dr. Pannell's laboratory. The instrument has electron impact, chemical ionization, fast atom bombardment and, recently electrospray ionization sources (the latter being purchased by LBC NHLBI). Most samples require integer mass analyses in the 500-2000 mass range by fast atom bombardment or accurate mass analyses for determination or proving of molecular formula or structure of natural products and synthetic samples.

A large expansion into the field of macromolecules has followed the installation of the high resolution mass spectrometer, the modification of the californium plasma desorption instrument for UV matrix assisted laser desorption ionization, and the installation of an electrospray ionization source. In collaboration with the Laboratory of Biophysical Chemistry, NHLBI, a macromolecular structure determination center is being established between NIDDK and NHLBI. This follows an increase in the awareness by biomedical investigators at NIH of the ability of mass spectrometry to solve problems in their fields and can be partly attributed to a biomedical mass spectrometry seminar series organized by LAC and LBC, NIDDK and LBC, NHLBI. Interfaces are being developed for the introduction of capillary zone electrophoresis and microbore LC separated samples into the electrospray source for identification and sequencing. It is estimated that next year about half of the project effort in high resolution mass spectroscopy will be concentrated in this area.

In collaboration with the National Institute of Standards and Technology, mass spectra of over 1200 compounds for which the structure has been elucidated at NIH have been obtained and processed for introduction to the NIST mass spectra library database. Samples are being obtained from many researchers at NIH in some cases involving samples that have been accumulated over 30 years of research here. A new mass spectrum is obtained and checked for purity and against the given structure of the compound. Only good spectra are forwarded to the NIST. The value of this database to mass spectrometrists world wide is immense, as any mass spectrometrist who identifies a new structure uses a search of this database to identify the compound if it is known, or to obtain information on functional pieces of an unknown structure. Very few of the compounds that have been isolated or synthesised at NIH over the years had been made available to the database before. This work is supported under a contract from NIST.

During the past year a large increase in simple load has occurred in Dr. Pannell's lab, with approximately 3200 sample being submitted for analysis (1700 in the previous year). A part time worker is to be employed to assist with the more routine samples.

Infrared Spectroscopy: A new Fourier transform infrared spectrometer (Digilab FTS-40) has been installed to provide the Laboratory with the capability to carry out efficient analyses of small amounts of sample. Mr. Whittaker serves as the manager of the instrument, which is designed for facile operation by individual investigators. The instrument is now in routine use.

Elemental Analysis: 850 samples were submitted to LAC for elemental analysis, involving 3540 separate analyses, primarily for carbon, hydrogen and nitrogen. Analyses are carried out via contract with two commercial laboratories. The number of samples is up substantially from the 500 submitted last year, and the number of individual analyses has tripled from FY 1990.

PUBLICATIONS

Yeh, H.J.C., Moody, E.J. and Skolnick, P. Halothane and anaesthesia, Nature, 346, 227 (1990).

Moody, E.J., Yeh, H.J.C. and Skolnick, P. The GABA receptor complex: is it a locus of action for inhalation anesthetics? in: Alcohol Reinforcement (Koob G, et al., eds.). Birkhauser, Boston (1990), pp 77-92.

Murata, Y., Yeh, H.J.C., Pannell, L.K., Jones, T.H., Fales, H.M. and Mason, R.T. New ketodienes from the integumental lipids of the Guam brown tree snake, Boiga Irregularis, J. Natural Products, 54, 233-240 (1991).

Sayer, J.M., Chadha, A., Agarwal, S.K., Yeh, H.J.C., Yagi, H. and Jerina, D.M. Covalent nucleoside adducts of benzzo[a]pyrene 7,8-diol 9,10-epoxides: structural reinvestigation and characterization of a Novel adenosine adduct on the ribose moiety., J. Org. Chem., 20-29 (1991).

Boye, 0., Yeh, H.J.C., Hamel, E., Wegrzynski, B., Toome, V. and Brossi, A. Antitubulin congeners of N-acetylcolchinyl methyl ether: synthesis of optically active 5-acetamidodeaminocolchinyl methyl ether and of demethoxy analogues of deamminocolchinyl methyl ether., J. Can. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58000-46 LAC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analytical Services and Methodology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. D. Becker	Acting Chief, Lab. Anal. Chem.	LAC/NIDDK
OTHER:	H. J. C. Yeh	Research Chemist	LAC/NIDDK
	N. Whittaker	Chemist	LAC/NIDDK
	W. White	Biologist	LAC/NIDDK
	L. K. Pannell	Research Chemist	LBC/NIDDK
	Gui-ying Li	Visiting Fellow	RCB,NCI
	H.L. Lee	Special Volunteer	LAC/NIDDK
	Quan-long Pu	Special Volunteer	LAC/NIDDK

COOPERATING UNITS *(if any)*

Laboratories of Bioorganic Chemistry, Medicinal Chemistry,Neuroscience, NIDDK
Radiation Oncology Branch NCI
Laboratory of Biophysical Chemistry, NHLBI

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Instrumentation Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER.
5	5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists, other NIH laboratories and, to a limited extent, personnel of other government agencies. Instrumental analyses include nuclear magetic resonance of liquids and solutions; infrared spectroscopy of solids, liquids, and samples introduced by gas chromatography; and mass spectrometry (with samples introduced by solids probe, gas and liquid chromtography and capillary zone electrophorsis with ionization by electron impact, chemical, electrospray, thermospray, therabeam, and fast atom bombardment.

Requests for analyses are obtained from many laboratories and Institutes at NIH, as well as some outside organizations. The laboratory in this way acts as an NIH resource and it is supported, in part, by its collaborators in terms of staffing and equipment where appropriate. Some major instruments are jointly funded and shared with other Institutes to avoid costly duplication of facilities.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58002-16

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Initial Intracellular Events of Steroid Hormone Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project transferred to LMCB 58002-16

U S GOVERNMENT PRINTING OFFICE 1991 0—864 939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 58003-17 LAC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Methods and Materials for the Study of Medical Problems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. M. Foltz	Research Chemist	LAC/NIDDK
OTHER:	B. Baer	Chemist	LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The primary goal of this work is to contribute to the investigation and solution of basic medical problems by the application of chemical, physical and biological methods. This goal is being pursued by studies of the biology and biochemistry of murine tumor cells with emphasis on cancer metastasis.

Studies were begun several years ago to determine whether one or more specific gene products are required to confer on certain tumor cells the properties needed for cancer metastasis. Promising preliminary results obtained with NIH 3T3 cells transfected with one of several oncogenes were reported previously. This work has been suspended temporarily because of other priorities.

Other lines of work in the area of cancer metastasis, such as the interactions of tumor cells with basement membrane components and other biological materials, the nutrition of tumor cells, and the effects of the treatment of tumor cells with chemicals and biologicals on metastatic potencies, are also to be resumed when the priorities imposed by other responsibilities permit. Meanwhile, and several lines of murine tumor cells, which have been used in the past and which are to be used in the future, are being maintained in mice and their metastatic potencies monitored.

During this period C. M. Foltz has been serving as NIDDK Safety Officer, Building 8/8A Occupant Emergency Coordinator and Safety Officer, and as Building 8/8A, Manager; much of his work has been in those areas.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 58004-24 LAC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Professional Practices of Biomedical Scientists

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Feder	Medical Officer (Research)	LAC/NIDDK
OTHER:	W. W. Stewart	Research Physicist	LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Analytical Chemistry

SECTION
Biophysical Histology

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies have continued on the professional practices of biomedical scientists and on the accuracy of the scientific literature.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 58005-17

PERIOD COVERED

Ocotber 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER.

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project transferred to LMC 59602-18

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER Z01-58007-07 LAC

PERIOD COVERED October 1, 1990 to September 30,1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)* Physostigmine and Analogs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

A.Brossi	Visiting Scientist	LAC/NIDDK
M.Brzostowska	Visiting Fellow	LAC/NIDDK
X.S. He	Visiting Fellow	LAC/NIDDK

COOPERATING UNITS *(if any)*

Drs.Nigel Greig and S.I.Rapoport,Laboratory of Neuroscience,NIA,NIH; Dr.Q.S.Yu, Institute of Organic Chemistry,Shanghai,China.

LAB/BRANCH Laboratory of Analytical Chemistry

SECTION Natural Products

INSTITUTE AND LOCATION NIDDK,NIH,Bethesda, Maryland 20892

TOTAL MAN-YEARS 1.0

PROFESSIONAL: 1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Optically active physovenines were prepared from (-)- and (+)-eseroline respectively. Carbamates of the (-)-series were found to be potent inhibitors of ChE in vitro. Appropriate modification of the procedure allowed the preparation of novel sulfur analogs of physovenines, with a sulfur atom replacing the oxygen atom in ring C.Carbamates of the thia-series were found to be extremely potent inhibitors of ChE in vitro. Phenylcarbamates of (-)-eseroline, substituted in the phenyl group with alkyl groups, afforded compounds which were highly specific in their actions in inhibiting either AChE, or BChE.

U.S. GOVERNMENT PRINTING OFFICE [illegible]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 58010-06 LAC

PERIOD COVERED
October 1,1990 to September 30,1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Mammalian Alkaloids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI..	A.Brossi	Visiting Scientist	LAC/NIDDK
Others	X.S. He	Visiting Fellow	LAC/NIDDK
	M.Brzostowska	Visiting Fellow	LAC/NIDDK
	D. Tadic	Visiting Fellow	LAC/NIDDK

COOPERATING UNITS (if any)
Laboratory of Bioorganic Chemistry (C. Creveling).

LAB/BRANCH
Laboratory of Analytical Chemistry (LAC).

SECTION Natural Products

INSTITUTE AND LOCATION
NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.8	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The biosynthesis of mammalian morphine from isoquinoline precursor alkaloids takes a different course from that in the opium poppy at the stage of (S)-3'-hydroxy-N-methylcoclaurine. This isoquinoline, on O-methylation with (S)-adenosylmethionine in the presence of mammalian catechol O-methyltransferase afforded almost exclusively (S)-orientaline, and not (S)-reticuline as required for further conversion into morphinan alkaloids and morphine.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 58011-15 LAC

PERIOD COVERED
October 1,1990 to September 30,1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Structure-Activity Relationships of Colchicinoids Based on Tubulin Binding

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI.	A.Brossi	Visiting Scientist	LAC/NIDDK
Others	O.Boye'	Visiting Fellow	LAC/NIDDK
	A.Muzaffar	Visiting Fellow	LAC/NIDDK

COOPERATING UNITS *(if any)*
L.Malspeis, LDDRD,National Cancer Institute; E.Hamel,LMP, National Cancer Institute; Dr.V.Simanek, Palacky University, Institute of Med. Chem.,Olomouc, Czechoslcvakia.

LAB/BRANCH
Laboratory of Analytical Chemistry

SECTION
Natural Products

INSTITUTE AND LOCATION
NIDDK,NIH,Bethesda,Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Novel isothiocyanates of thiocolchicine and colchicine allo-congeners were prepared as potential markers of the colchicine binding site on tubulin. The most interesting compound is 9-isothiocyanato-9-deoxy-N-acetylcolchinol which will be prepared in radiolabeled form to ensure specificity.Analogs of the potent inhibitor N-acetylcolchinyl methyl ether lacking methoxy groups at C(l), or at C(9), were found to be not very active. Several compounds of the colchicine series which showed good in vitro activity against human tumor cell lines will be tested in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK 58014-03

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Project Transferred to LMC 59601-04

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-58017-02 LAC

PERIOD COVERED October 1,1990 to September 30,1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nortropane Alkaloids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

A.Brossi	Visiting Scientist	LAC/NIDDK
X.S.He	Visiting Fellow	LAC/NIDDK

COOPERATING UNITS (if any) National Institutes of Pharmaceutical Research & Development, Beijing,China (Dr.C.C.Shen).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION Natural Products Section

INSTITUTE AND LOCATION

NIDDK,NIH,Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Efficient synthesis of several N-protected 6-acetoxynortropin-3-ones has been achieved.N-Benzyl-6-acetoxynortropin-3-one was converted into 6-acetoxynortropane. Bromination of 6-acetoxytropin-3-one and its N-protected noranalogs afforded mixtures of 2- and 4-brominated ketones.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 58018-02 LAC

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analytical Reagents from Dihydroflourescein

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.,	A.Brossi	Visiting Scientist	LAC/NIDDK
Others	M.Brzostowska	Visiting Fellow	LAC/NIDDK
	D. Tadic	Visiting Fellow	LAC/NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH Laboratory of Analytical Chemistry

SECTION Natural Products

INSTITUTE AND LOCATION NIDDK,NIH,Bethesda Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1-Phenylethylamides of O-methyldihydrofluorescein were obtained in form of two optically pure diastereomers. This is scientific proof that dihydrofluoresceins with differently substituted aromatic rings in the xanthene moiety can exist as optical isomers. Such compounds may be useful to recognize portions of biopolymers.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-[illegible]

ANNUAL REPORT OF THE LABORATORY OF NEUROSCIENCE

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Studies on the benzodiazepine/GABA receptor chloride channel complex

The benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") is an oligomeric group of proteins that contain recognition sites for many psychopharmacological agents including benzodiazepines, ß-carbolines, barbiturates, ethanol and inhalation anesthetics. The proteins comprising this complex act in concert to regulate the activity of chloride channels that are controlled ("gated") by gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the vertebrate central nervous system. Studies are in progress to characterize the molecular aspects of this system, its physiological functions and possible role in disease.

The $GABA_A$ receptor complex appears to be one member of a "superfamily" of ligand-gated ion (both cation and anion) channels which may include channels gated by glycine, acetylcholine, and glutamate. While the $GABA_A$ complex in mammalian central nervous system is currently thought to be constituted by at least three distinct but related classes of proteins (termed alpha, beta and gamma), neither the number nor arrangement of subunits required to form a fully functional drug and ligand-gated chloride channel is known. Based on the large number of possible subunit combinations available to form a fully functional $GABA_A$ receptor complex, it was postulated that the pharmacological actions of a benzodiazepine depend in part on receptor (BzR) ligand (that is, ranging from agonist [diazepam-like] to inverse agonist [3-carbomethoxy-ß-carboline-like] would depend in part on receptor conformation. The observation that Ro 15-4513 behaves as a "GABA-neutral" (antagonist) ligand at "diazepam-insensitive" BzR and as a "GABA-negative" (inverse agonist) ligand at "diazepam-sensitive" BzR is consistent with the hypothesis that subunit composition determines not only ligand affinity but also ligand efficacy.

Previous studies from this laboratory have demonstrated that many of the neurological manifestations in two different animal models of hepatic encephalopathy (HE) can be blocked by administration of benzodiazepine receptor antagonists such as Ro 15-1788 (flumazenil). In addition, extracts from the brains of these models contain elevated concentrations of diazepam and N-desmethyldiazepam. The demonstration that brain concentrations of these 1,4-benzodiazepines were significantly elevated in a subpopulation of patients who succumbed from fulminant hepatic failure are consistent with previous findings in animal models of hepatic encephalopathy, and provides a rational basis for the use of BzR antagonists in the management of hepatic encephalopathy.

Studies on glycine and glutamate coupled cation channels

We previously demonstrated that the glycine partial agonist 1-aminocyclopropanecarboxylic acid (ACPC) mimics the effects of clinically effective anxiolytics in both conflict (elevated plus-maze) and non-conflict (ultrasonic vocalization in rat pups) animal models. In addition, ACPC, a cation channel blocker (MK-801) and a competitive NMDA antagonist (AP-7) to mimic clinically effective antidepressants in two animal models. These observations suggest that glutamatergic pathways may be involved in the pathophysiology of affective disorders. The findings that ACPC is effective following oral administration in these paradigms and that its action is long-lived following either oral or parenteral administration indicate further long-term experiments are necessary to demonstrate efficacy and safety. The demonstration that the methyl ester of ACPC is more potent than the parent compound indicates that derivatives with even greater potency can be synthesized. The development of a precise and sensitive analytical method for the measurement of ACPC will be invaluable for the study of the pharmacokinetic and pharmacodynamic factors contributing to its actions.

Studies on neural-immune interactions

We previously demonstrated that administration of anxiogenic ß-carbolines are immunosuppressive, implicating the $GABA_A$ receptor complex in the neural control of immunity. Further studies on the molecular mechanisms of action indicate these actions may be mediated via the hypothalamic-pituitary-adrenal axis, and are independent of both the sympathetic nervous system and opiate receptors.

Using a simple and reliable method for dual-color analysis of heterogenesis cell populations, it was previously shown that inhibition of calcium influx into splenocytes may be an early event in opiate-induced immunosuppression. The demonstration that stress-induced immunosuppression also involves changes in calcium mobilization indicates changes in cytosolic free calcium may be a common mediating diverse types immunosuppressive phenomena.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK,58501-05 LNS

PERIOD COVERED

October 1, 1990 - September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Receptors for Neurotransmitters & Drugs in Brain & Peripheral Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: P. Skolnick, Chief, LN, NIDDK. Others: P. K. Arora, Expert; A.S. Basile, Senior Staff Fellow; K. Boje, Staff Fellow; G.E. Evoniuk, Guest Researcher; Z-Q, Gu Guest Researcher; R. Maity, Visiting Fellow; R. Munherjee, Special Volunteer; R.T. McCabe, Special Volunteer; R. Miller, Chemist; G. Nowak, Visiting Fellow; I.A. Paul, Guest Researcher; Y. Sei, Visiting Associate; G. Wong, Guest Researcher

COOPERATING UNITS *(if any)* E.A. Jones, DDB, NIDDK; K. Rice, B. DaCosta, LMC, NIDDK; K. Jacobsen, LBC, NIDDK; H. Yeh, LAC, NIDDK; M.B.H. Youdim, H. Pollard, G. Kuypers, LCMBG, NIDDK; H. Morse, J. Hartley, M. Makino, LVD, NIAID; L. Vitkovic, LI, NIAID; J. Chader, T. Fletcher, J. Raber, LVR, NEI; (cont'd.)

LAB/BRANCH

Laboratory of Neuroscience

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Section on Neurobiology

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NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11.2	11.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

High affinity, stereospecific recognition sites (receptors) for neurotransmitters, neuromodulators, and many clinically useful drugs have been identified in both peripheral tissues and the central nervous system. The interaction of a neurotransmitter, neuromodulator or drug with a specific recognition site initiates a series of events (for example, the opening of an ion channel or activation of an enzyme) resulting in either a physiological response (in the case of a neurotransmitter or neuromodulator) or a pharmacological effect (in the case of a drug). Furthermore, the presence of recognition sites for synthetic substances indicates that endogenous substances may also be present which can mimic (or antagonize) the effects of exogenously applied (synthetic) compounds. Studies are in progress to characterize "recognition-effector" systems, to link novel recognition sites to effector systems, to develop appropriate model systems, and to relate these systems to both physiological and pathological processes

ANNUAL REPORT OF THE MOLECULAR PATHOPHYSIOLOGY BRANCH
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate normal and abnormal cell function at the molecular level with emphasis on transmembrane signalling by hormones, neurotransmitters, growth factors, and other first messengers acting at the cell surface. Approaches used range from molecular biologic techniques to clinical investigation in an effort to define the pathogenesis of diseases characterized by abnormal signal transduction.

Guanine nucleotide binding proteins (G-proteins) as receptor-effector couplers

A family of G-proteins functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for polypeptide hormones, monoamine neurotransmitters, photons of light, chemical odorants, chemotactic factors, and certain growth factors. Effector functions regulated by G-proteins include cAMP formation, cGMP degradation, phosphoinositide breakdown, and several types of ion channel. Major areas of interest concerning G-proteins include: 1)definition of the diversity within this gene family; tissue and subcellular distribution; regulation of gene expression. 2)definition of domains on individual G-protein subunits involved in association of the subunits, attachment to cell membranes, interaction with receptor and effector domains, and possible interactions with other regulatory proteins. 3)definition of the degree and mechanism of specificity for individual G-proteins in coupling to both receptors and effectors. 4)definition of quantitative and qualitative alterations in G-proteins that result in altered signal transduction. Significant recent progress has been made in each of these areas:

1) Molecular basis for subunit association and membrane attachment of GTP-binding proteins- G-proteins are heterotrimers; alpha subunits reversibly associate with a beta/gamma complex. The holoprotein is associated with the cytoplasmic side of the plasma membrane, but the basis for membrane attachment and the domains responsible for subunit association have not been defined. By metabolic labeling of transfected cells and immunoprecipitation of expressed alpha subunits, we can show that Gi but not Gs-alpha subunits undergo a specific fatty acid acylation (myristoylation). Site-directed mutagenesis of the myristoylation site has been performed. The mutant alpha subunit fails to be myristoylated, and upon transfection in cos cells, remains in the cytosol, unlike the wild-type which is targeted to the cell membrane. This highlights the critical role of this covalent modification in membrane attachment. The basis for Gs-alpha attachment remains unclear. Extensive mutagenesis studies indicate that neither the extreme amino- nor carboxy-termini are critical for Gs-alpha membrane attachment.

The basis for membrane association of the beta/gamma complex has also been defined. The gamma subunit ends in a "C-A-A-X" motif and has been shown to undergo posttranslational processing similar to that of the ras oncogene product, i.e. proteolytic cleavage of the last three amino acids, carboxymethylation of the resultant cysteine, and isoprenylation via the cysteine sulfhydryl. Elucidation of this pathway has important implications, since inhibition of this processing would preclude attachment of the beta/gamma complex. Under native conditions, beta and gamma subunits are tightly but noncovalently associated. We now find that expression of either beta or gamma subunits in cos cells is dependent on cotransfection of both cDNAs. This implies that a beta/gamma complex is formed early after translation and is necessary for expression of these subunits. Mutagenesis of the gamma subunit CAAX cysteine to serine blocks prenylation. This leads to expression of beta/gamma heterodimer in the cytosol of transfected cos cells. The results indicate that CAAX processing is critical for beta/gamma membrane targeting, but not for stable heterodimer formation. [Drs.Jones, Juhnn, Murakami, Simonds, Collins, and Spiegel, in collaboration with Dr. Backlund]

2) Altered G-proteins as a cause of altered signal transduction- As critical intermediates in the signal transduction pathway, quantitative or qualitative alterations in G-proteins could have a major impact on the signalling process. We have generated mutant alpha subunits for several G proteins that should lead to either constitutive activation or dominant inhibition of G protein function. The mutant alpha subunits have been initially characterized after transient expression in cos cells. Mutant Gi2-alpha cDNAs have been stably transfected into NIH 3T3 cells. The constitutively active mutant causes increased cell proliferation, while the dominant inhibitory mutant slows growth. [Drs. Merendino, Hermouet, and Spiegel]

3) Specificity of G protein receptor-effector coupling- We have made specific peptide antibodies against novel G protein alpha subunits. As with previous antisera we raised against the carboxy-termini of alpha subunits, the new reagents are capable of uncoupling receptor from G protein in native membranes, and can be used to define the specificity of coupling. Studies in human platelet membranes show that an antibody against the novel Gq-alpha protein uncouples the thromboxane A2 receptor, whereas the Gi2-alpha-specific antibody blocks alpha-2-adrenergic stimulation. The Gq antibody defines a novel class of pertussis toxin-insensitive G protein, presumptively linked to phospholipase C stimulation. [Drs. Shenker, Goldsmith, and Spiegel, in collaboration with Dr. Unson]

Pseudohypoparathyroidism (PHP)

PHP is a genetic disorder in which resistance to parathyroid hormone (PTH) may be associated with somatic abnormalities collectively termed Albright's hereditary osteodystrophy (AHO). We have previously shown that subjects with this form of PHP are resistant to multiple hormones that act by stimulating cAMP formation, that an approximate 50% reduction in activity of the G-protein (Gs) that couples receptors to stimulation of adenylyl cyclase is present in all tissues from affected subjects, and that subjects with PHP show reduction in steady state mRNA for the Gs-alpha subunit. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects. This work indicates that mutations in a G protein gene can lead to clinically significant disease. [Drs. Weinstein, Friedman and Spiegel, in collaboration with Dr. Gejman]

McCune-Albright syndrome (MAS)

MAS is a non-genetic disorder in which affected subjects show a variety of seemingly unrelated abnormalities including polyostotic fibrous dysplasia, pigmented skin lesions (cafe-au-lait spots), and autonomous hyperfunction of various endocrine organs including gonads, anterior pituitary, thyroid, and adrenal cortex. The endocrine abnormalities lead to precocious puberty, gigantism, hyperthyroidism, and hypercortisolism. The cause of this sporadic disorder has been completely enigmatic, but speculations have centered on a defect in signal transduction leading to endocrine hyperfunction. The distribution of skin lesions has also suggested the possibility of a somatic mutation acquired early in embryogenesis and affecting only a subset of cells (mosaicism). Since a G protein mutation could plausibly explain the endocrine manifestations, we searched for and found mutations of the Gs-alpha gene that lead to constitutive activation of the Gs protein. These mutations were found in a mosaic distribution; notably, mutant gene was undetectable in normal-appearing portions of endocrine glands, but was present at heterozygous levels in neoplastic portions of endocrine tissue. Our studies suggest that MAS is caused by a somatic mutation in the Gs-alpha gene occurring early in development and found in a mosaic distribution. [Drs. Weinstein, Shenker and Spiegel, in collaboration with Dr. Gejman]

Molecular biologic studies on the cause of parathyroid neoplasia

Parathyroid tumors (benign adenomas, hyperplasia, and carcinoma) are presumptively due to acquired (and in some cases such as multiple endocrine neoplasia type I {MEN I} to inherited) abnormalities at the gene level. We are studying the molecular basis for parathyroid neoplasia by searching for mutations, rearrangements and/or deletions in genomic DNA from parathyroid tumors. We have found rearrangement of the parathyroid hormone gene in only 1 of 43 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In contrast, point mutations in ras oncogenes were not found in any parathyroid tumors. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus on 11q13 may encode a tumor "suppressor" gene. By mapping deletions at 11q13 in parathyroid tumors, we are attempting to identify the MEN I gene.

Honors and Awards:

Lee Weinstein (Senior Staff Fellow) was invited to present his work at the International Serono Symposium: "Endocrinology under 35."

Teresa Jones (Medical Staff Fellow) was invited to present her work at the Institute for Molecular Biology, Moscow, USSR.

Eitan Friedman (Visiting Associate) was invited to present his work at an international meeting on "Hereditary Tumors" in Florence, Italy.

James Butrynski (HHMI-NIH research scholar) and Andrew Shenker (PRAT fellow) won young investigator travel awards to the American Federation for Clinical Research National Meeting in Seattle.

Paul Goldsmith (Research Chemist) was invited to speak at the Jacques Monod Conference on signal transduction in Assois, France.

Allen Spiegel (Branch Chief) was invited to lecture at National and International Meetings including the 18th Aharon Katzir-Katchalsky Conference in Italy, the American College of Neuropsychopharmacology Annual Meeting, the Gordon Conference on second messengers, and the 7th International Symposium on Cellular Endocrinology. He also received the Jacobaeus Prize of the Nordisk Insulin Foundation and delivered the prize lecture in Oslo, Norway.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DK 59000-04 MPB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Molecular biologic studies on the cause of parathyroid neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK
Others: E. Friedman, M.D., Visiting Associate, MPB, NIDDK

COOPERATING UNITS *(if any)*
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G. Aurbach, M.D. Chief, MDB, NIDDK
J. Norton, M.D., Chief, Surg. Metab. Section, Surgery Branch, NIDDK

LAB/BRANCH
Molecular Pathophysiology Branch

SECTION

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NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Primary hyperparathyroidism (HPT), a common endocrine disorder that can cause significant morbidity, may be due to benign neoplasia of a single parathyroid gland (adenoma) or multiple parathyroid glands (hyperplasia), and rarely, to malignant neoplasia of a parathyroid gland (carcinoma). The etiology of parathyroid neoplasia has not been defined. As with other forms of neoplasia, parathyroid tumors are presumably due to inherited (germ-line mutation) and/or acquired (somatic mutation) defects in specific genes. Etiologic genetic defects could include inappropriate expression of transforming "oncogenes" and/or loss of expression of tumor "suppressor" genes. The availability of surgically resected parathyroid tumors from patients with sporadic and hereditary forms of disease allows us to search for tumor-specific genetic abnormalities that may be involved in development of parathyroid neoplasia. We have found rearrangement of the parathyroid hormone gene in only 1 of 43 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In contrast, point mutations in ras oncogenes were not found in any parathyroid tumors. In both "hyperplastic" glands from subjects with inherited multiple endocrine neoplasia type I (MEN I) and in sporadic adenomas, loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus in 11q13 may encode a tumor "suppressor" gene. We are attempting to identify the MEN I gene among other methods by mapping deletions at 11q13 in parathyroid tumors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 59001-26 MPB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Guanine nucleotide binding proteins as receptor-effector couplers

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others: T. Jones, M.D., Med. Staff Fellow, MPB, NIDDK
P. Goldsmith, Ph.D., Res. Biol., MPB, NIDDK; N. Thambi, Ph.D., Visit. Fell.
W. Simonds, M.D., Sen. Staff Fellow, MPB, NIDDK
Yung-Sung Juhnn, M.D., Visit. Fell., MPB, NIDDK
J. Merendino, M.D., Med. Staff Fell., MPB, NIDDK
S. Hermouet, M.D., Visit. Fell., MPB, NIDDK; A. Shenker, M.D., Med. Staff.
T. Murakami, M.D., Visit. Fell., MPB, NIDDK

COOPERATING UNITS *(if any)*

C. Unson, Rockefeller Univ., N.Y.: P. Backlund (NIMH)

LAB/BRANCH

Molecular Pathophysiology Branch

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NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11	9	2

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A family of guanine nucleotide binding proteins (G-proteins) functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for hormones, neurotransmitters, light, odorants, and certain growth factors. Effector functions regulated (positively and, in some instances, negatively) by G-proteins include cAMP formation, phosphoinositide breakdown, potassium and calcium channels, and cGMP degradation. We have used a variety of techniques to study the expression, distribution, regulation, structure and function of G-proteins. Our studies highlight the diversity within the G-protein family. Using peptide specific antibodies, we have defined the specificity of G-proteins in coupling to receptors and effectors. We have defined distinct post-translational lipid modifications necessary for membrane attachment of G protein alpha and beta/gamma subunits. We have created mutations in alpha subunits that cause constitutive activation, and transfected these into cells to define phenotypic effects on cellular function. These studies provide the basis for understanding the role of G-proteins in normal signal transduction and for elucidating possible defects in G-protein structure or function as the basis for abnormal signal transduction.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 59002-26 MPB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on pseudohypoparathyroidism and related disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK

Others: L. Weinstein, M.D., Medical Staff Fellow, MPB, NIDDK
E. Friedman, M.D., Visiting Associate, MPB, NIDDK

COOPERATING UNITS (if any)

P. Gejman, Clin. Neurogenetics Branch, NIMH

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism" (PHP). Patients with this disorder show characteristic constitutional features (Albright's hereditary osteodystrophy - AHO) and do not respond to exogenous parathyroid hormone (PTH). In PHP, UcAMP (urinary cyclic AMP) does not increase normally in response to PTH administration. This indicates that there is a defective hormone receptor-adenylate cyclase complex in this disorder. We have shown that many patients with PHP+AHO (PHP Ia) show an approximately 50% reduction in activity of Gs (the stimulatory guanine nucleotide binding protein associated with adenylate cyclase) in membranes from multiple tissues. Gs deficiency presumably accounts for resistance to multiple hormones in such patients. Using cloned human cDNA probes for the alpha subunit of Gs, we have shown that steady state mRNA levels from fibroblasts of subjects with PHP Ia are reduced by approximately 50% compared with normals. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 59003-01 MPB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on McCune-Albright Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A.Spiegel, M.D., Chief, MPB, NIDDK
Others: L.Weinstein, M.D., Medical Staff Fellow, MPB, NIDDK
A.Shenker, M.D., Ph.D., Medical Staff Fellow, MPB, NIDDK

COOPERATING UNITS *(if any)*

P. Gejman, Clin. Neurogenetics Branch, NIMH

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

McCune-Albright syndrome (MAS) is a non-genetic disorder in which affected subjects show a variety of seemingly unrelated abnormalities including polyostotic fibrous dysplasia, pigmented skin lesions (cafe-au-lait spots), and autonomous hyperfunction of various endocrine organs including gonads, anterior pituitary, thyroid, and adrenal cortex. The endocrine abnormalities lead to precocious puberty, gigantism, hyperthyroidism, and hypercortisolism. The cause of this sporadic disorder has been completely enigmatic, but speculations have centered on a defect in signal transduction leading to endocrine hyperfunction. The distribution of skin lesions has also suggested the possibility of a somatic mutation acquired early in embryogenesis and affecting only a subset of cells (mosaicism). Since a G protein mutation could plausibly explain the endocrine manifestations, we searched for and found mutations of the Gs-alpha gene that lead to constitutive activation of the Gs protein. These mutations were found in a mosaic distribution; notably, mutant gene was undetectable in normal-appearing portions of endocrine glands, but was present at heterozygous levels in neoplastic portions of endocrine tissue. Our studies suggest that MAS is caused by a somatic mutation in the Gs-alpha gene occurring early in development and found in a mosaic distribution.

The following summary describes selected advances made by the three Sections in LMC during 1990-1991.

DRUG DESIGN AND SYNTHESIS SECTION

Opioid Receptors

The unequivocal identification of saturable, enantioselective, high affinity mu, delta and kappa opioid receptor subtypes in the mammalian central nervous system (CNS) has resulted from many converging lines of chemical, pharmacological and biochemical investigation. In addition, evidence continues to accumulate supporting the existence of similar receptor subtypes on components of the immune system. The structure of these subtypes and their function in modulation of the CNS and immune system are extremely important questions and are currently the subject of intense investigation in many laboratories. The significance of such studies has recently been heightened by concerns regarding the relationship of opiate induced immunosuppression to the spread of acquired immune deficiency syndrome (AIDS) in intravenous narcotic abusers. Central to the success of our past and ongoing studies of receptor subtype structure and function has been the synthesis and identification of (a) selective high affinity ligands for each subtype (b) irreversible ligands with receptor subtype specificity and (c) enantiomeric pairs of these ligands for detection of receptor mediated effects.

Pharmacological Properties of (+)-Buprenorphine and (+)-Diprenorphine. Both (+)-buprenorphine and (+)-diprenorphine were synthesized from (+)-thebaine. These (+)-isomers were obtained from the NIH Opiate Total Synthesis. (-)-Buprenorphine and (-)-diprenorphine are semisynthetic opium derivatives with established pharmacological profiles produced by interaction with classical opioid mu, kappa, and delta receptors. (-)-Buprenorphine is a potent analgesic which demonstrates narcotic agonist-antagonist properties and (-)-diprenorphine is a narcotic antagonist capable of reversing the agonist actions of pure agonists and some mixed agonist-antagonists. More recently, (-)-buprenorphine has been found to suppress cocaine self-administration in monkeys through mechanisms which are unclear at this time. Although the unnatural (+)-opiate enantiomers traditionally have insignificant affinity for classical opioid receptors in comparison to their counterparts, some of these compounds have been found to bind to other CNS sites and could have potential clinical utility. For example, the (+)-opiate-related dextromethorphan displays neuroprotective properties in CNS ischemic injury models and is a nonprescription antitussive with low affinity for the classical opioid receptors. Examination of the pharmacological profile of the (+)-isomers of buprenorphine and diprenorphine is therefore important both for defining the role of the (-)-isomers at the opioid receptor and in the search for new therapeutic agents. In addition, (+)-buprenorphine is a potentially valuable research tool in mechanistic studies of suppression of cocaine self-administration by the (-)-isomer. Our results indicate that mu, kappa, and delta binding sites exhibit affinity in the picomolar range for the (-)-isomers of buprenorphine and diprenorphine. Conversely, as expected, none of these receptors possesses high affinity for either of the (+)-isomers. The opioid receptor subtype binding enantioselectivity ratio [Ki(+)/Ki(-)] exceeds 1,000,000 for these compounds. These results are important for evaluating the safety and specificity of these compounds as potential medicinal agents (e.g., antitussives). Previously, the preference of the mu receptor for (-)- over (+)-etorphine (Ki ratio = 350,000) was thought to be one of the most extreme examples of receptor enantioselectivity. However, the delta receptor demonstrates an extremely high relative affinity for (-)-diprenorphine in comparison to (+)-diprenorphine (Ki ratio = 70,000,000).

The enantioselectivity of the delta receptor for (-)- over (+)-diprenorphine far exceeds that of the mu receptor for (-)- over (+)-etorphine and is the largest enantioselectivity ratio of which we are aware.

Oxide-Bridged 5-Phenylmorphans as Probes for Narcotic Receptor Mediated Phenomena. 5-(m-Hydroxyphenyl)-2-methylmorphan (1) was designed and synthesized by Dr. E. L. May 35 years ago in NIDDK. It has proven to be a compound with a remarkable pharmacological profile. In its racemic form the compound shows morphine-like activity, but although both the (+)- and (-)-enantiomers possess strong antinociceptive activity, the (-)-antipode is not morphine-like in a number of in vivo tests, suggesting its interaction with different (non-mu) opioid receptors. Compound 1 is conformationally heterogeneous. The three low-energy conformers, typified by the phenyl ring eclipsing each of the three C-C bonds, are not equally populated. Introduction of a methyl group in the 9-alpha position restricts the freedom of rotation and the number of accessible conformations even more, and leads to decreased agonist and increased antagonist activity. In order to correlate the pharmacological activities of 5-phenylmorphans with their conformations (i.e., the orientation of the phenyl ring relative to the bicyclo[3.3.1]nonane skeleton) the torsion angle phi was fixed by anchoring the aromatic moiety with an epoxy bridge. Two sets of six compounds can be envisaged, having the oxide bridge either ortho or para relative to the other oxygen function. Connection of the epoxy bridge to positions a, d, and f of the bicyclo-[3.3.1]nonane part of the molecule afforded compounds which showed relatively low affinity for the opioid receptors (IC50=1766, 1000, and 96 nM, respectively, compared with the IC50 of 1 = 5.2). We have now begun a study directed towards the synthesis of the ortho- and para-substituted "e"-isomers. A ketone intermediate was synthesized from 2-(m-methoxyphenyl)cyclohexanone in four steps. Stereoselective reduction to the alcohol, followed by partial reduction of the aromatic nucleus gave the cyclohexadiene. Preliminary experiments suggest that hydrolysis to the conjugated ketone and bromination with N-bromoacetamide led to formation of the desired oxide bridge. Aromatization will give the desired 5-phenylmorphan. Depending on the conditions used for the hydrolysis, it may be possible to convert to a non-conjugated ketone, which can serve as starting material for the ortho-substituted isomer.

Computer-assisted molecular modeling of phenylmorphans. As discussed above, 5-(m-hydroxyphenyl)-2-methylmorphan (1) and derivatives show an interesting spectrum of activities ranging from potent analgesia to moderate narcotic antagonism, depending both on the absolute configuration and on substituents at C-9. In part, these different activities may be linked to the occurrence of different conformations. Restriction of the rotation by a bridging unit, such as the oxygen in the oxide-bridged phenylmorphans, defines the spatial relationship between the orientation of the nitrogen lone pair, the phenolic OH, and the aromatic ring. These structural data can be used to describe a pharmacophore and relate receptor affinity and selectivity to a conformation. Molecular mechanics calculations on 1 show that the rotational barriers and the differences in energy between the conformers of 1 are too low to be biologically relevant. It is noteworthy that the X-ray conformation is significantly different from any of the minimum-energy conformations of 1. Receptor points, representing hydrophobic interaction with the aromatic ring and a hydrogen bond to the nitrogen, were built onto the minimum-energy conformations of 1, as well as a potent antagonist with an SAR pattern similar to 1, and various isomers. When overlayed, the f-isomer (a weak antagonist) and also the, yet unsynthesized, c-isomer show an excellent fit with energy minima of 1 and the potent antagonist. The a- and d-isomers, which have low affinity for opiate receptors, show close similarity to high-energy conformations of 1.

Epimeric Oxide Bridged 5-(m-Hydroxyphenyl)morphans as Probes for Narcotic Receptor Mediated Phenomena. As part of this study on the opioid receptor system we have undertaken the synthesis of a series of conformationally restricted, oxide-bridged 5-(m-hydroxyphenyl)morphans, in order to relate their conformation to the agonist/antagonist properties shown by nonbridged analogs. We have approached the synthesis of the two epimeric compounds para "c" and "d" isomers, through the common intermediate enamine 5-(2,5-dimethoxyphenyl)azabicyclo[3.3.1]nonene. Construction of the enamine was accomplished by the application of the method of Evans and Zimmerman. Bromination, followed by elimination of HBr and reduction of the double bond afforded a single bromo epimer, which was converted to the para "d" isomer by O-demethylation with BBr3 and base-mediated epoxy ring closure. Also, the nucleophilic displacement of the bromide obtained from the enamine was accomplished by treatment with potassium benzoate which afforded the benzoate ester with inversion of configuration. Hydrolysis to the corresponding alcohol followed by esterification gave the unstable mesylate. O-Demethylation with BBr3 under forcing conditions, followed by work-up with aqueous KOH resulted in the predominant formation of an undesired tetrahydropyran derivative and traces, in variable yields, of a compound whose spectroscopic properties suggest the desired para "c" isomer. Work is in progress on the improvement of the reaction conditions required for the formation of this compound.

The Affinity Ligand 'UPHIT' Acylates the Kappa Opioid Receptor In Vivo. The development of irreversible antagonists represents a significant advancement in the characterization of opioid receptors. Our recently synthesized UPHIT has been found to irreversibly inhibit binding at kappa sites. Effects seen are both kappa-specific and enantioselective, as demonstrated by the lack of change in labeling of mu and delta sites and by the relative ineffectiveness of the (1R,2R)-enantiomer in inhibiting kappa binding. Furthermore, UPHIT preferentially reduces binding at the kappa site labeled by [3H]U69,593. The negligible reduction in the Bmax values indicates that the elimination of [3H]U69,593 binding does not reflect occupancy of these sites. Its enantioselectivity is indicative of initial binding by UPHIT to [3H]U69,593 sites; the greatly elevated Kd suggests that UPHIT may produce a conformational change in the site, thereby increasing the subsequent dissociation af agents. UPHIT is a valuable addition to the already existing selective irreversible antagonists, and may prove useful in determining the biological function of kappa receptor subtypes. We have also synthesized high specific activity tritium labelled 1S,2S-trans-2'-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide, a specific irreversible ligand for kappa opioid receptors. Optically pure tritium labeled 1S,2S-(-)-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, an affinity ligand specific for the kappa opioid receptor was synthesized from optically pure 1S,2S-(-)-trans-2-amino-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzenacetamide via the sequence of dibromination (57%) followed by catalytic tritiation of the dibromide. The resulting tritium labelled aniline (14% yield, specific activity 31.2 Ci/mmol) was transformed to the desired compound in 13.3% yield and 99+% radiochemical purity by treatment with thiophosgene.

Probing the Opioid Receptor Complex With (+)-trans-SUPERFIT. Evidence That [D-Pen2,D-Pen5]Enkephalin Interacts With High Affinity at the delta-cx Binding Site. A variety of data support the existence of an opioid receptor complex composed of distinct but interacting mu-cx and delta-cx binding sites, where "cx" indicates "in the complex." The ability of subantinociceptive doses of [Leu]enkephalin and [Met]enkephalin to potentiate and attenuate morphine-induced antinociception, respectively is thought to be mediated via their binding to the delta-cx binding site. [D-Pen2,D-Pen5]Enkephalin also modulates morphine-induced antinociception, but has very low affinity for the delta-cx binding site in vitro. In the present study, membranes were depleted of their delta-ncx binding sites by pretreatment with the site-directed acylating agent, (3S,4S)-(+)-trans-N-[1-[2-(4-isothiocyanato)phenyl)-ethyl]-3-methyl-4-piperidyl]-N-phenylpropaneamide hydrochloride, which permits selective labeling of the delta-cx binding site with [3H][D-Ala2,D-Leu5]enkephalin. The major findings of this study are that with this preparation of rat brain membranes: a) there are striking differences between the delta-cx and mu binding sites; and b) both [D-Pen2,D-Pen5]enkephalin and [D-Pen2,L-Pen5]enkephalin exhibit high affinity for the delta-cx binding site.

The Potent Opioid Agonist, (+)-cis-3-Methylfentanyl Binds Pseudoirreversibly to the Opioid Receptor Complex In Vitro and In Vivo: Evidence For a Novel Mechanism of Action. We have demonstrated that pretreatment of rat brain membranes with (+)-cis-3-methylfentanyl {(+)-cis-MF}, followed by extensive washing of the membranes, produces a wash-resistant decrease in the binding of [3H]-[D-ala2,D-leu5]enkephalin to the delta binding site of the opioid receptor complex (delta-cx binding site). Intravenous administration of (+)-cis-MF (50 micrograms/kg) to rats produced a pronounced catalepsy and also produced a wash-resistant masking of delta-cx and mu binding sites in membranes prepared 120 min post-injection. Administration of 1 mg/kg i.v. of the opioid antagonist, 6-desoxy-6-beta-fluoronaltrexone (cycloFOXY), 100 min after the injection of (+)-cis-MF (20 min prior to the preparation of membranes) completely reversed the catatonia and restored masked delta-cx binding sites to control levels. This was not observed with (+)-cycloFOXY synthesized via the NIH Opiate Total Synthesis. The inability of cycloFOXY to restore masked delta-cx binding sites in vitro is consistent with other studies. The in vivo data suggest that the antagonist binds to the receptor, and facilitates the dissociation of the agonist, possibly through an allosteric mechanism. These data further suggest that the mu and the delta-cx binding sites are not the same and that the binding of (+)-cis-MF to the opioid receptor complex produces catalepsy in the rat. (+)-cis-MF may produce a conformational change in the receptor complex facilitating coupling to intramembrane effector proteins and producing a long-lasting effect at low fractional occupancy of receptors.

RTI-4614-4: An Analog of (+)-cis-3-Methylfentanyl with a 27,000-Fold Binding Selectivity for mu versus delta Opioid Binding Sites. The Ki values (nM) of an analog of (+)-cis-3-methylfentanyl, racemic cis-N-[1-(2-hydroxy-2-phenylethyl)-3-methyl-4-piperidyl]-N-phenylpropanamide HCl (RTI-4614-4) for opioid receptor subtypes were as follows: mu (0.0055), delta (148), kappa1 (84.8), kappa2a (2275), and kappa2b (22.3). The selectivity of this agent for the mu binding site was 27,000 vs. the delta binding site, 15,400 vs. the kappa1 binding site, 413,700 vs the kappa2a and 4,054 vs the kappa2b binding site. In contrast, two other fentanyl analogs, N-(2-(4-methylpyridinyl))-N-(1-phenethyl-4-piperidinyl)2-furamide and N-(2-pyrazinyl)-N-(1-phenethyl-4-piperidinyl) 2-furamide had considerably higher Ki values at, and were less selective for, the mu binding site. Since RTI-4614-4 is composed of a mixture of four stereoisomers, the resolution of these isomers should permit identification of an extremely potent and selective agent for the opioid mu receptor.

Pseudoirreversible Binding of Opiates to Opioid Receptor Subtypes in Rat Brain: A Quantitative Study. Morphine, naloxone, fentanyl, (+)-cyclazocine, and (-)-cis-MF at 1 micromolar did not act as pseudoirreversible inhibitors of mu or delta binding sites. However, lofentanil, (+)-cis-MF, ohmefentanyl (OHM) and sufentanil all produced a dose-dependent pseudoirreversible inhibition of mu binding sites. They had no effect on delta binding sites. Saturation binding studies using control and membranes pretreated with 500 nM OHM demonstrated that the pseudoirreversible inhibition of [3H]OHM binding by this agent was due to a 7-fold increase in the Kd, not a decrease in the Bmax, a result compatible with the presence of residual drug. To directly address the issue of residual drug, membrane suspensions were centrifuged, rather than filtered, and the supernatants assayed for inhibitory activity in the binding assay. Membranes pretreated with 500 nM OHM had enough inhibitory activity to account for a 2-fold increase in the Kd, suggesting that residual drug plays a minor role in the pseudoirreversible increase of the Kd of mu binding sites. Dissociation experiments compared the dissociation of [3H]OHM from membranes pretreated with 0 nM (control membranes) or 500 nM ohmefentanyl (OHM-membranes). [3H]OHM dissociated so slowly from control membranes that a dissociation rate constant could not be calculated. However, [3H]OHM dissociated measurably faster from OMH-membranes. Viewed collectively, these data suggest that the fentanyl analogs produce pseudoirreversible inhibition of mu receptor binding via an allosteric mechanism. During the preincubation phase of the protocol, the drugs bind tightly to an allosteric site, which might be, for example, a subdomain of the mu receptor. This produces a conformational change in the mu receptor, which is detected as an increase in the Kd and an increase in the dissociation rate. Since the fentanyl analogs which produce pseudoirreversible inhibition are also considerably more potent in tests of antinociception than their apparent affinity for the mu receptor predicts, these data suggest that their capacity to bind to this allosteric site might contribute to their extraordinary potency in vivo.

Interaction of beta-Funaltrexamine With [3H]CycloFOXYBinding in Rat Brain: Further Evidence That beta-FNA Alkylates the Opioid Receptor Complex. beta-Funaltrexamine (beta-FNA) is an alkylating derivative of naltrexone. In addition to acting as an irreversible inhibitor of mu-receptor-mediated physiological effects, intracerebroventricular (i.c.v.) administration of beta-FNA to rat attenuates the ability of selective mu receptor antagonists and naloxone to reverse mu receptor-mediated effects. Moreover, recent work demonstrated that i.c.v. administration of beta-FNA alters the conformation of the opioid receptor complex, as inferred by a decrease in the Bmax of the lower affinity [3H][D-ala2,D-leu5]enkephalin binding site. Consistent with the decreased potency of naloxone as an inhibitor of mu receptor mediated effects, beta-FNA doubled the naloxone IC50 for displacing [3H][D-ala2,D-leu]enkephalin from its lower affinity binding site. These data collectively support the hypothesis that the opioid receptor complex postulated to mediate mu interactions in vivo is identical to the opioid receptor complex as defined by in vitro ligand binding studies. A direct prediction of this hypothesis is that beta-FNA should increase the Kd of antagonists for the mu binding site (mu-cx) of the receptor complex. The data reported in this paper has found that beta-FNA doubled the IC50 of the potent narcotic antagonist, 6-desoxy-6-beta-fluoronaltrexone (cycloFOXY) for displacing [3H][D-ala2,D-leu5]enkephalin from its lower affinity binding site, and doubled the Kd of [3H]cycloFOXY for its mu binding site, providing additional data that the mu binding site labeled by [3H]cycloFOXY is the mu binding site of the opioid receptor complex. beta-FNA also altered the kappa binding site labeled by [3H]cycloFOXY, and when administered intrathecally to mice, beta-FNA produced a long lasting antinociception in the acetic acid writhing test.

Upregulation of the Opioid Receptor Complex By the Chronic Administration of Morphine: A Biochemical Marker Related to the Development of Tolerance and Dependence. Studies conducted after the development of the rapid filtration assay for opiate receptors, and before the recognition of multiple opioid receptors, failed to detect changes in opioid receptors induced by chronic morphine. Recent experiments conducted in our laboratories were designed to examine the hypothesis that only one of several opioid receptor types might be altered by chronic morphine. Using binding surface analysis and irreversible ligands to increase the "resolving power" of the ligand binding assay, the results indicated that chronic morphine increased both the Bmax and Kd of the opioid receptor complex, labeled with either [3H][D-Ala2,D-Leu3]enkephalin, [3H][D-Ala2-MePhe4, Gly-ols]enkephalin or [3H]6-desoxy-6-beta-fluoronaltrexone. In the present study rats were pretreated with drugs known to attenuate the development of tolerance and dependence [the irreversible mu-receptor antagonist, beta-FNA, and the inhibitor of tryptophan hydroxylase, *para*-chlorophenylalanine], prior to subcutaneous implantation of morphine pellets. The results demonstrated that 1) unlike chronic naltrexone, beta-FNA failed to upregulate opioid receptors and 2) both beta-funaltrexamine and PCPA pretreatment attenuated the chronic morphine-induced increase in the Bmax but not the Kd, of the opioid receptor complex. These results provide evidence that naltrexone-induced upregulation of the opioid receptor complex might occur indirectly as a consequence of interactions at beta-funaltrexamine-insensitive opioid receptors and that morphine-induced upregulation (increased Bmax) of the opioid receptor complex is a relevant in vitro marker related to the development of tolerance and dependence. These data collectively support the hypothesis that endogenous anti-opiate peptides play an important role in the development of tolerance and dependence to morphine.

Upregulation of Rat Brain Opioid Receptors By the Chronic Administration of Morphine: Possible Evidence For an Anti-opiate Model of Tolerance and Dependence. The mechanism(s) underlying the development of tolerance and dependence to opiates has been the subject of intensive investigation for several decades. The experimental systems used include clonal cell lines, smooth muscle bioassay systems and whole animal studies. These various investigations have identified several potentially interactive mechanisms which include acute desensitization, receptor down-regulation, altered receptor interactions with G proteins and the generation of spare receptors. The consistent finding of a chronic morphine-induced increase in the Bmax of the delta-cx binding site, an effect similar to that observed in rats chronically administered antagonists such as naltrexone, prompted speculation that the administration of morphine resulted in a secretion of endogenous antagonists, which like naltrexone, produce an increase in the Bmax of the opioid receptors. Consistent with this hypothesis, the CNS synthesizes and secretes peptides which are known to attenuate the actions of morphine. Some of these peptides, such as beta-endorphin, dynorphin and met5-enkephalin, presumably produce this attenuation via direct interactions at opioid receptors. Other peptides, such as alpha-MSH, tyr-MIF, MMP-8 (Phe-Leu-Phe-GlnPro-Gln-Arg-Phe-NH2), CCK-8 and glucagon presumably functionally attenuate actions of morphine via interactions at their own specific receptor. These peptides, which we generically refer to as "anti-opiate" peptides, a term describing the ability of the peptides to attenuate the actions of opiates, were suggested to participate in the development of tolerance and dependence. According to the anti-opiate model of tolerance and dependence, the continuing administration of morphine results in increasing levels of anti-opiates, which attenuate the effects of morphine, thereby maintaining homeostasis. Administration of an exogenous antagonist, such as naloxone, abruptly disturbs this homeostasis, resulting in the withdrawal syndrome. Synergism between exogenous and endogenous antagonists account for the observation that the more tolerant the animal, the less naloxone is required to trigger withdrawal. Finally, the anti-opiates upregulate the delta-cx binding site, much as administration of an exogenous antagonist would. An attractive aspect of the anti-opiate model of tolerance and dependence, is that it generates several testable predictions: 1) administration of morphine should increase CSF levels of anti-opiates; 2) i.c.v. administration of anti-opiates to a tolerant/dependent animal should trigger withdrawal; 3) i.c.v. administration of antibodies directed against anti-opiates should prevent development of tolerance and dependence; 4) i.c.v. administration of antibodies directed against anti-opiates should prevent the morphine-induced increase in the Bmax of opioid receptors; 5) chronic administration of anti-opiates should increase the Bmax of the same opioid receptor subtypes upregulated by chronic morphine. A discouraging aspect of the anti-opiate model of tolerance and dependence is that there are so many peptides which potentially act as anti-opiates. Clearly, criteria must be established for deciding which anti-opiate peptides might play pivotal roles in tolerance and dependence, and therefore be appropriate candidates for additional research. It was felt that a peptide satisfying predictions 1-3 would be a suitable candidate for further study. In view of recent data demonstrating that 1) CSF levels of MMP-8 increase during chronic administration of morphine; 2) administration of MMP-8 to tolerant/dependent rats triggers withdrawal; 3) that administration of anti-MMP-8 IgG to tolerant/dependent rats prevents naloxone-induced withdrawal and 4) that MMP-8 has its own CNS binding site, it was felt that MMP-8 more than satisfied the criteria mentioned above. Our experiments begin to examine predictions 4 and 5. Perhaps the most interesting finding of this work is that chronic administration of anti-MMP IgG produces a substantial increase in the Bmax of [3H]DAGO binding sites without altering the Kd. Since the IgG probably does not readily penetrate into the brain from the ventricular space, these data support the hypothesis that MMP-8 in the CSF exerts a tonic negative control over the the density of [3H]DAGO binding sites. This interpretation is at odds with the results obtained from chronic i.c.v. infusion of MMP-8. Clearly, further experiments, including direct measurement of the MMP-8 receptor, will be required to address this paradox. Unlike the prediction of the anti-opiate model that administration of anti-MMP IgG would prevent the morphine induced increase in the Bmax, both chronic morphine and chronic anti-MMP IgG increased the [3H]DAGO Bmax, but the effects were not additive. It seems possible, however, that perhaps another anti-opiate peptide might be responsible for mediating this effect. The observation that anti-MMP-8 IgG partially attenuated the ability of chronic morphine to increase the Kd suggests that MMP-8 may partially contribute to this particular effect of chronic morphine.

In Vivo Pharmacology of Potent, mu-Selective Opioids: Variations in Opioid Efficacy. We have examined the in vivo pharmacology of potent, mu-selective opioids and have found variations in opioid efficacy. Several fentanyl-related 4-(heteroanilido)-piperidines with interesting pharmacological effects have been reported in the literature. One of these derivatives (1) was studied under a variety of conditions in rhesus monkeys and compared to other mu opioids (e.g., alfentanil) as well as to a second fentanyl derivative (mirfentanil). Compound 1 and mirfentanil maintained rates of self-administration responding comparable to rates maintained by alfentanil; these positive reinforcing effects of compound 1 and mirfentanil were attenuated by quadazocine. Compound 1 substituted completely and mirfentanil substituted only partially for alfentanil in monkeys discriminating between saline and alfentanil; neither compound 1 nor mirfentanil substituted for ethylketocyclazocine in monkeys discriminating between ethylketocyclazocine and saline. In morphine-treated monkeys discriminating between naltrexone and saline, mirfentanil but not compound 1 substituted for naltrexone. In morphine-abstinent monkeys compound 1 but not mirfentanil completely reversed naltrexone-lever responding. This withdrawal-reversing effect of compound 1 was attenuated in a dose-related manner by opioid antagonists; Schild analyses of these antagonisms indicated mu-receptor mediation of this effect of compound 1. Both compound 1 and mirfentanil had analgesic effects in a warm water tail-withdrawal assay; this analgesic effect of compound 1 was an opioid effect as evidenced with antagonism by quadazocine. In contrast, the analgesic effect of mirfentanil under these conditions appeared to be non-opioid. Compound 1 but not mirfentanil markedly decreased respiratory function in monkeys breathing air or 5% $CO2$ in air. The respiratory-depressant effects of compound 1 were antagonized by quadazocine. In contrast, mirfentanil antagonized the respiratory effects of other mu opioids (e.g., alfentanil). Compound 1 is structurally very similar to mirfentanil; however, the small difference in structure between compound 1 and mirfentanil appears to confer a significant increase in efficacy for compound 1 at opioid mu-receptors. Further studies on the structure-activity relations of these and other fentanyl derivatives might provide important information on the molecular requirements of opioid efficacy.

Delineation of Central Opioid Receptors Regulating Natural Killer Cell Activity In Vivo. We have found that opiate-induced suppression of natural killer (NK) cell activity is mediated primarily through mu-type opioid receptors. Information on the opioid receptor-subtype involved is important in predicting and controlling the outcome of opioid administration on immune function in vivo. Research in this area could ultimately lead to the logical design and development of potent opioid analgesics which do not compromise NK cell function. Such agents could be useful in the treatment of pain in burn victims and cancer patients, as well as many other clinical situations where suppression of the immune system would not be desired. Opiates have well-documented immunosuppressive properties. We have presented evidence that the inhibition of natural killer cell (NK) activity produced by morphine in vivo is centrally mediated and primarily involves the periaqueductal grey area. In order to determine the CNS opioid receptor subtypes responsible for opiate effects on NK cytotoxicity, agonists selective for the major classes of opioid receptors were microinjected into the lateral ventricle of Fischer 344N male rats. Three hours after injection, splenic NK activity was determined using a 5 hour chromium release assay. Dose ranges of 20-200 nmol of (S,S)-U50,488 and and 60-200 nmol of [D-Pen-2,5]-enkephalin (DPDPE), selective for kappa and delta receptors, respectively, did not affect NK cytotoxicity. The lowest DPDPE dose, 20 nmol, increased this measure. In contrast, 60-200 nmol of the selective mu agonist [D-Ala2, NMe-Phe4, glyol]-enkephalin (DAGO) caused a reduction in NK activity, blocked by pretreatment with naltrexone (5 mg/kg, i.p.). These findings indicate that opiate-induced immunosuppression is mediated primarily through central mu receptors. DAGO (60 nmol, i.c.v.) had no effect on plasma corticosterone or ACTH levels three hours postinjection, suggesting that central mu binding does not reduce NK activity through activation of the hypothalamic-pituitary adrenocortico-axis. The fentanyl derivative mirfentanil produces analgesia in rats without respiratory depression and has mixed agonist-antagonist effects at mu receptors. Mirfentanil (40 mg/kg, s.c.) did not affect NK activity; however, pretreatment with mirfentanil blocked the severe reduction in NK activity induced by sufentanil (0.06 mg/kg, s.c.), a pseudoirreversible mu agonist. These preliminary data provide evidence that analgesia and immunosuppression produced by mu agonists are mediated by distinct binding sites or binding states. Mirfentanil is an example of new possibilities in the design of analgesics having neither respiratory-depressive nor immunosuppressive effects.

The Committee (now, College) on Problems of Drug Dependence (CPDD) Drug Testing Program. In vitro and in vivo assays have been used to study new analgesics, stimulants and depressants, under the auspices of the College on Problems of Drug Dependence, with the goal of determining their physical dependence potential and abuse liability. Data from the studies on stimulants and depressants have been utilized by the Expert Committee of the World Health Organization which is charged with the evaluation of scientific data used for drug scheduling under the Psychotropic Substances Convention. The CPDD is now a World Health Organization Collaborating Center for research and training in the field of drug dependence.

Golden Anniversary Celebration of NIDDK Intramural Research on Drugs of Abuse and the Continuing Interaction with the Committee on Problems of Drug Dependence 1941-1991

This one day symposium was held during the annual meeting of the CPDD and co-sponsored by NIDDK. It was co-organized by Drs. Jacobson and Rice and moderated by Dr. Andrew Thurkauf. The following speakers and topics were selected to outline major advances during the period and discuss their impact on contemporary medicinal chemistry/neuroscience and the implications for future advances in this area.

A. E. Jacobson, LMC, NIDDK: "The Continuing Interrelationship of CPDD and NIDDK"
A. H. Newman, Addiction Research Center, NIDA, Baltimore, MD: "Potent Analgesia, Respiratory Depression, Dependence, Abuse Liability - Clue to Separability"
E. L. May, Medical College of Virginia, Richmond, VA: "Leads to Current Therapy, to Opioid Receptor Subtypes, and to Sigma Receptors"
S. Archer, Rensselaer Polytechnic Institute, Troy, NY: "State-of-the-Art Analgesics from the Agonist-Antagonist Concept"
W. Klee, LMB, NIMH: "Essential Ligands for Discovery and Characterization of Opioid Receptor Subtypes"
B. de Costa, LMC, NIDDK: "Novel Kappa Opioid Receptor and Sigma Receptor Ligands"
A. E. Jacobson, LMC, NIDDK: "A New Generation of Electrophilic Affinity Ligands for the Phencyclidine Binding Sites"
R. Johnson, Glaxo Inc., Research Triangle Park, NC: "The Cannabinoid Receptor - Pharmacological Identification, Anatomical Localization, and Cloning"
K. C. Rice, LMC, NIDDK: "Opiate Total Synthesis and Contemporary NIDDK Analgesic Research - Natural and Unnatural Ligands for Computed Tomography Imaging of Receptors in Conscious Humans. Implications for Future Advances in the Neurosciences"
L. S. Harris, Medical College of Virginia, Richmond, VA: "Concluding Remarks - Pharmacology"
J. W. Lewis, Reckitt and Colman, England, UK: "Concluding Remarks - Chemistry"

This symposium was recorded as a television quality videotape production to allow future generations of researchers to see and hear the historical and contemporary research upon which so much of the present and future research in the area is, or will be based. One or more editions of the videotape are intended to create a greater awareness of the research which has been accomplished, and that which is ongoing, in intramural NIDDK, and perhaps more importantly, to serve as a powerful future recruitment tool for young researchers. The videotapes will be distributed to universities with departments and students involved in the neurosciences, nationally and internationally, upon request.

A number of interesting "then and now" contrasts emerged from the presentations which served to dramatically illustrate progress in research on drugs of abuse. Dr. Newman discussed the first concerted medicinal chemical, pharmacological and clinical efforts to separate the pharmacological effects of morphine during 1929-1941 which strongly suggested such a goal could be accomplished. Dr. Rice contrasted these studies, which utilized relative primitive tools and techniques by standard of today, with development of the NIH Opiate Total Synthesis, an example of determining biochemical function of opiate receptors in living animals and imaging such receptors in conscious humans by positron emission tomography. Dr. Archer summarized analgesic development from the agonist-antagonist concept to the present which has largely validated the prediction of the

1929-1941 efforts. The comments by a number of world renowned researchers during the symposium and the concluding remarks by Drs. Harris and Lewis, which provided a lively conclusion to the symposium, set the stage for stimulating discussions for the remainder of the meeting. It was generally agreed the symposium was excellent, that much has been accomplished and much remains to be done. With regard to the latter, the consensus was that good medicinal chemistry and rational drug design will be even more essential to neuroscience in the coming century as more drug receptors are cloned, the exact structure of their combining sites determined and as methods for manipulation of the corresponding signaling systems are elucidated.

Site Directed Affinity Ligands for GABA-gated Chloride Channels

Novel Site-Directed Affinity Ligands For GABA-gated Chloride Channels: Synthesis, Characterization and Molecular Modelling of 1-(Isothiocyanatophenyl)-4-(tert-butyl)-2,6,7-trioxabicyclo[2.2.2]octanes. In our work on novel site-directed affinity ligands for GABA-gated chloride channels, we have synthesized, and carried out molecular modeling of 1-(isothiocyanatophenyl)-4-tert-butyl-2,6,7-trioxabicyclo-[2.2.2]octanes. p-(1), m-(2), and o-Isothiothiocyanate (3) derivatives of tert-butylbicycloorthobenzoate (TBOB) were synthesized from 3-tert-butyloxetane-3-methanol as the starting material. While the meta-compound was readily obtained in four steps via catalytic hydrogenation of the m-nitro-tert-butylbicycloorthobenzoate intermediate, the ortho and para isomers could not be obtained this way. They were instead synthesized by an alternative four-step approach which made use of the stability of the isothiocyanate moiety to strong Lewis acids such as boron trifluoride etherate, conditions that would isomerize isothiocyanato oxetane ester intermediates to their corresponding orthoesters. The p-isothiocyanate derivative of TBOB (1) inhibited [35S]-tert-butylbicyclophosphorothionate (TBPS) binding to rat cortical membranes with a potency (IC50=62 nM) comparable to the parent compound while 2 and 3 were approximately 10-fold less potent (IC50=570 and 609 nM, respectively). Preincubating tissue with radioligand further reduced the potencies of 2 and 3 about 10 fold (IC50=5400 and 7500 nM, respectively) while the potency of I (IC50=90 nM) was only marginally affected by this procedure. Pretreatment of membranes with I and 2 followed by extensive washing resulted in a concentration-dependent inhibition of [35S]TBPS binding. In contrast, preincubating tissues with up to 2.4 micromoles of 3 did not elicit an apparent acylation of [35S]TBPS binding sites. Molecular modeling of the effective diameters of 1-3 in their thermodynamically most stable conformations indicated a relationship between these diameters and their relative efficacies as site-directed acylators; the smaller the diameter, the more potent the acylator. This hypothesis explains both the relative potencies of these compounds and their differential abilities to acylate the TBPS binding site.

Studies Towards the Development of a Cocaine Antagonist

High Affinity Dopamine Reuptake Blockers as Potential Cocaine Antagonists: A Strategy For Drug Development. The addictive and euphorogenic effects of cocaine are thought to result primarily from inhibition of dopamine reuptake. Although the potency of cocaine-like drugs as inhibitors of DA reuptake is highly correlated with their potency as reinforcers in animals, several potent DA reuptake blockers (bupropion, nomifensine, benztropine, and mazindol) have not been reported to produce addiction or euphoria in humans. Based on these observations in humans, DA reuptake inhibitors are classified into two groups; type 1 blockers, which produce addiction and euphoria, and type 2 blockers, which do not. Given that type 1 and type 2 blockers act at the same site (the DA transporter), we suggest that type 2 agents may antagonize the effects of cocaine, and might prove useful in the treatment of cocaine addiction.

Evidence for High and Low Affinity [3H]Cocaine Binding Sites Associated With the Serotonin Reuptake Complex in Guinea Pig Brain: Allosteric Modulation By Paroxetine. We have determined that the high and low affinity [3H]cocaine binding sites are associated with the serotonin reuptake complex in guinea pig brain. [3H]Cocaine binding was specific and saturable. Binding surface analysis resolved two binding sites, site 1 (Kd=15.2 nM, Bmax=183 fmol/mg protein) and site 2 (Kd=650 nM, Bmax=1880 fmol/mg protein). Cocaine had highest affinity for site 1, and paroxetine had the highest affinity for site 2. The potent DA reuptake inhibitors mazindol, benztropine and GBR12909 had low affinity for both site and site 2, and GBR12935 had unexpectedly high affinity for site 2. Viewed collectively, these data suggest that [3H]cocaine labels two binding sites associated with the serotonin transporter in membranes of whole guinea pig brain. The two sites could represent two states of a single receptor, two distinct binding sites associated with a single class of transporters, or perhaps two sites, each associated with a different class of transporters. The observation that paroxetine pseudoirreversibly inhibits [3H]cocaine binding via an allosteric mechanism suggests that paroxetine binds tightly to a site which allosterically controls the conformation of the [3H]cocaine binding site.

Actions of (+)-Buprenorphine On Cocaine and Opiate-Mediated Effects. The Partial Opioid Agonist, Buprenorphine, Protects Against Lethal Effects of Cocaine. The role of classical opiate receptors in cocaine addiction is now being intensely studied. We have formerly determined the binding profile of (+)-buprenorphine, one of the invaluable research tools available from the NIH Opiate Total Synthesis, at classical opioid receptors. Because this isomer exhibits no affinity for opiate receptors, (+)-buprenorphine is useful as a control in studies of antinociception and "kappa abstinence syndrome", a behavior precipitated collectively by (-)-buprenorphine, naloxone, and nor-BNI. Our studies in these areas reveal that the antinociceptive properties of buprenorphine can be effectively measured using the rat paw formalin test and that the (+)-isomer exhibits no activity in this model. In addition, (+)-buprenorphine does not induce kappa abstinence syndrome under conditions where the (-)-isomer precipitates various stereotypic behaviors associated with this syndrome. Recently, (-)-buprenorphine has been found to produce dose-dependent protection against the lethal effects of cocaine in mice. (+)-Buprenorphine did not protect the rats up to doses more than 100 times the lowest effective dose of its (-)-enantiomer. Low doses of naltrexone (0.3—1.0 mg/kg) blocked the protective effects of (-)-buprenorphine. Protection conferred by buprenorphine was not observed in CXBK mice, a recombinant inbred strain relatively devoid of mu-opioid receptors. Thus, buprenorphine appears to protect against the lethal effects of cocaine by a process which could be mediated by mu-opioid receptors. The present results should provide some additional safety assurance in future clinical trials with buprenorphine, especially in outpatient trials where cocaine abuse may continue along with treatment. Future clinical studies will be necessary to more fully evaluate the utility of buprenorphine in the treatment of cocaine addiction.

Phencyclidine Recognition Sites

We have studied the action of phencyclidine (PCP)-like ligands on glutamate receptors of the N-methyl-D-aspartate (NMDA) type. Phencyclidine binding sites have been implicated as allosteric sites which interact with glutamate receptors of the NMDA type. Some phencyclidine (PCP)-like compounds have recently been reported to exert a robust protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. Other sites for interaction with PCP-like ligands in the CNS have also been found, including the dopamine uptake complex.

Three Generations of Electrophilic Affinity Ligands for Phencyclidine Binding Sites. Phencyclidine (PCP)-like ligands are known to bind within excitatory amino acid controlled ion channels regulated by the NMDA/glutamate receptor. These ligands act as non-competitive antagonists and block excessive influx of endogenous ligands and ions through the channel induced by traumatic events such as head injury or stroke. By this blockade, PCP-like ligands such as MK-801 (Dizocilpine) are thought to exert their neuroprotective effects. PCP-like ligands also interact with the dopamine transporter complex to block dopamine reuptake. We have prepared three generations of electrophilic affinity ligands with increasing potency and selectivity for the PCP binding sites. Our third generation irreversible ligand was based on MK-801, one of the most potent PCP-like drugs. We have now synthesized two MK-801 isothiocyanates, one of which has proven to be selective and more potent in its interaction with the PCP binding sites than our former affinity ligands. It irreversibly blocks half of the binding sites labeled by [^{3}H]MK801 at a 100 nM concentration and it appears to interact minimally with the dopaminergic reuptake system. We have also redetermined the PCP pharmacophore with MK-801 using a Silicon Graphics 4D70GT minicomputer and the Polygen Quanta and CHARMm software, and have contrasted the preferred conformation of MK-801 with other PCP-like compounds. The observed differences may help rationalize the interaction of PCP-like compounds with at least two sites in the brain, and the relative inactivity of MK-801 at one of these sites.

Pseudoallosteric Modulation by (+)-MK801 of NMDA-Coupled Phencyclidine Binding Sites. Two high affinity phencyclidine (PCP) binding sites, labelled by [3H]1-[1-(2- thienyl)cyclohexyl] piperidine ([3H]TCP), have been identified in guinea pig brain, with one site coupled to the N-methyl-D-aspartate (NMDA) receptor (site 1) and the other site associated with the dopamine reuptake carrier complex (site 2). In this study, PCP enhanced the dissociation of [3H]TCP from PCP site 1 and site 2, while (+)-MK801 only enhanced dissociation of [3H]TCP from PCP site 1. Although additional studies will be required to determine the exact mechanism(s) of these effects, these data demonstrate that the interactions of PCP with both site 1 and site 2 are more complex than previously appreciated.

Characterization of NMDA-Coupled and Dopamine Reuptake Carrier Coupled [^{3}H]TCP Binding Sites in Guinea Pig Brain. We have conducted kinetic experiments to evaluate the modulation of the MK-801-sensitive (site 1) and MK-801-insensitive (site 2) binding sites by NMDA receptor agonists and antagonists and tested the hypothesis that site 2 is associated with the DA reuptake carrier in guinea pig brain. Evaluation of the association of PCP site 2 with the DA reuptake carrier involved BTCP and GBR12909, two potent inhibitors of DA reuptake. Neither drug had any significant effect on PCP site 1, except 10,000 nM BTCP, which inhibited [3H]TCP binding to this site by 20%. BTCP produced a dose-dependent wash-resistant inhibition of [3HITCP binding to PCP site 2 at concentrations as low as 100 nM (20% inhibition), while GBR12909 required higher concentrations (1000 nM, 46% inhibition). The kinetic experiments demonstrated that glutamate, a NMDA receptor agonist, Mg ion, a noncompetitive NMDA receptor antagonist and AP7, a competitive NMDA receptor antagonist modulated [3H]TCP binding to PCP site 1 but not PCP site 2, which suggests that PCP site 1 is coupled to the NMDA receptor complex. Preincubation of guinea pig striatal membranes with varying concentrations of the high affinity DA reuptake inhibitors, GBR12909 and BTCP, caused a dose-dependent, wash-resistant inhibition of [3H]TCP binding to PCP site 2 but not PCP site 1. This tight binding phenomena suggests that PCP site 2 is associated with the DA reuptake complex. These data raise the possibility that PCP's behavioral effects might be separately mediated via the two binding sites: the psychotomimetic effects could be mediated via the DA uptake carrier site, since an increase in the release of DA has been associated with psychotic behavior, and the therapeutic properties could be linked to PCP's noncompetitive inhibition of NMDA-receptor mediated effects.

Synthesis and Evaluation of [3H]-4-Fluoro-1-[1-(3-hydroxyphenyl)cyclohexyl]piperidine. A Potential Tool For Autoradiographic Study of the Phencyclidine Receptor. [18F]4-Fluoro-1-[1-(2-thienyl)cyclohexyl)]piperidine is a ligand potentially useful for positron emission tomography of the PCP/NMDA receptor complex in the mammalian brain. The tritium-labelled compound was synthesized in 5 steps starting with cyclohexanone. Catalytic tritiolysis in the final step afforded the desired tritiated compound in high radiochemical yield and specific activity. This method represents a more efficient approach than previously published syntheses. We have also described the synthesis and in vitro binding of high specific activity tritium labelled 4-fluoro-1-[1-(3-hydroxyphenyl)cyclohexyl]piperidine ([3H]FOH-PCP), a potential probe for autoradiographic study of the PCP receptor. [3H]FOH-PCP will enable evaluation of $[^{18}F]$FOH-PCP as a PET scanning ligand for PCP receptors.

Cannabinoid Receptors

Cannabinoid Receptor Localization in Brain. [3H]CP 55,940, a radiolabeled synthetic cannabinoid, which is 10-100 times more potent in vivo than delta9-tetrahydrocannabinol, was used to characterize and localize a specific cannabinoid receptor in brain sections. The potencies of a series of natural and synthetic cannabinoids as competitors of [3HICP 55,940 binding correlated closely with their relative potencies in several biological assays, suggesting that the receptor characterized in our in vitro assay is the same receptor that mediates behavioral and pharmacological effects of cannabinoids, including human subjective experience. Autoradiography of cannabinoid receptors in brain sections from several mammalian species, including human, reveals a unique and conserved distribution; binding is most dense in outflow nuclei of the basal ganglia—the substantia nigra pars reticulata and globus pallidus—and in the hippocampus and cerebellum. Generally high densities in forebrain and cerebellum implicate roles for cannabinoids in cognition and movement. Sparse densities in lower brainstem areas controlling cardiovascular and respiratory functions may explain why high doses of delta9-tetrahydrocannabinol are not lethal.

Sigma Receptor Ligands

Sigma receptors are non-dopaminergic, non-opioid receptors which bind PCP- and D2-dopamine antagonist-related compounds, as well as some of the (+)-enantiomers of the morphinan and benzomorphan opioids. Studies utilizing various sigma ligands have implicated sigma receptors in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. Although progress is being made in elucidating the physiological roles of sigma receptors, the biochemical systems modulated by sigma receptors are not clear.

Synthesis and Receptor Binding of Enantiomeric N-Substituted cis-N-[2-(3,4-Dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamines As High Affinity Sigma Receptor Ligands. We have identified a novel class of highly potent sigma ligands related to the kappa-selective agonist U50,488. Although the functional role of sigma receptors is not fully understood, they have been implicated in the mediation of physiological effects which include the regulation of motor behavior, the dystonic side-effects of antipsychotic drugs as well as in neuroprotection in ischemia models. We have recently shown that the cis diastereomers of the kappa-selective agonist U50,488 are potent ligands for sigma receptors with Ki values of 81 and 221 nM respectively, in competition with [3H]-(+)3-PPP. However, these drugs exhibited weak affinity for kappa receptors. This observation led us to further investigate their structure-activity relationships. A systematic structure-activity study was performed involving the synthesis of more than 40 compounds. This was done by varying the arylacetyl moiety and leaving the rest of the molecule unchanged. The strategy followed was first to test the racemic analogs for their affinity at kappa receptors. The enantiomers of the most potent and selective ligands of the series were then examined for their binding at sigma, kappa, PCP and D2-dopamine receptors. Of the analogs examined, the compound with the highest affinity for sigma receptors was a diamine. Its 1R,2S-(-)-enantiomer, BD737, showed a Ki=1.3 nM and the 1S,2R-(+)-enantiomer, BD738, had a Ki=6 nM. Both of them had very little or no affinity for kappa, PCP and D2-dopamine receptors. We have shown that the cis-N-[2-(1-pyrrolidinyl)cyclohexyl] arylethylamines exhibit high affinity for sigma receptors. A better understanding of the functional role of these receptors may be possible through use of this new class of sigma ligands.

A Practical Synthesis, Optical Resolution and Determination of Absolute Configuration of Enantiomerically Pure 1S,2R-(+)- and 1R,2S-(-)-cis-2-(1-Pyrrolidinyl)cyclohexyl amines: Important Precursors For a New Class of Sigma-Receptor Ligands and Anticonvulsant Drugs. We have now obtained important precursors for this new class of sigma-receptor ligands and anticonvulsant drugs through the practical synthesis, optical resolution and determination of the absolute configuration of enantiomerically pure 1S,2R-(+)- and 1R,2S-(-)-cis-2-(1-pyrrolidinyl)cylcohexylamines. The enantiomerically pure compounds were prepared starting with racemic trans-2-aminocyclohexanol. The key step in the generation of the cis stereochemistry of these compounds employed catalytic hydrogenation of the enamine mixture generated from condensation of pyrrolidine with racemic 2-(benzamido)cyclohexanone. An efficient optical resolution of the racemate was achieved through two recrystallizations of the mandelate salts. The absolute configuration of the (+)- and (-)-enantiomers was assigned as 1S,2R and IR,2S, respectively, based on conversion of the (-)-cis-2-(1-pyrrolidinyl)cyclohexylamine to, and comparison with the N-methyl derivative of known 1R,2S absolute configuration.

Synthesis and Receptor Binding of Enantiomeric N-Substituted cis-N-[2-(3,4-Dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamines As High Affinity Sigma Receptor Ligands. For our work on high potency sigma ligands, we have synthesized and determined the receptor binding of enantiomeric N-substituted cis-N-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamines. N,N-Alkyl-substituted derivatives of (+)- and (-)-cis-N-[2-(3,4-dichlorophenyl)ethyl]-2(1-pyrrolidinyl)cyclohexylamine have been synthesized in nine steps in a stereospecific manner starting from cyclohexene oxide. The key step in the reaction sequence involved catalytic hydrogenation of an oxime in the presence of PtO2 and AcOH to give the racemic cis diamine. Most of the compounds in this series exhibited very high affinity at the sigma receptor when tested against [3H]-(+)-3-PPP, and in general it was observed that the IR,2S enantiomers bound more potently to sigma receptors than their corresponding IS,2R enantiomers. The most potent sigma ligand found in this class was the unsubstituted IR,2S-(-)-derivative, which exhibited an affinity constant of 0.49 nM. This compound was also found to be very selective for the sigma receptors. It exhibited little or no affinity for kappa opioid, PCP, and dopamine-D2 receptors. It was also demonstrated that the cis configuration in this series was necessary for high sigma receptor affinity.

Synthesis, Characterization and Biological Evaluation of a Novel Class of Conformationally Unrestricted N-[Arylethyl]-N-alkyl-2-(1-pyrrolidinyl)ethyl amines: Structural Requirements and Binding Affinity at the Sigma Receptor. By synthesizing and testing a part-structure, N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine (1), derived from our previously reported high affinity sigma receptor ligands 1S,2R-(-)-N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)cyclohexylamine [(-)-2 and (+)-2], we have identified a novel class of superpotent (subnanomolar affinity) sigma ligands specific for the sigma receptor labelled by [^{3}H]-(+)-3-PPP. When 1 was tested for its capacity to displace [^{3}H]-(+)-3-PPP from guinea pig brain membranes, it exhibited a Ki of 0.34 nM which is better than either of its parent compounds (-)-2 (Ki=1.3 nM) and (+)-2 (Ki=6.0 nM). The synthesis of 1 was readily accomplished in 3 steps starting with commercially available 1-(2-chloroethyl)pyrrolidine; the sequence of methylaminolysis (65%), DCC mediated coupling with 3,4-dichlorophenylacetic acid (98%) and alane (AlH_3) reduction in THF (74%) afforded the target compound 1. By changing the primary amine used in the aminolysis step, a number of different side-chain analogs of 1 were obtained in good yield. Changing the nature of the acid in the DCC coupling step allowed evaluation of the effect of aromatic substitution. Other compounds related to 1 such as N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-homopiperidinyl)ethylamine (Ki=0.17 nM) were synthesized starting with N-t-Boc protected glycine and homopiperidine; the sequence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride mediated coupling (71%), $LiAlH_4$ reduction (80%), DCC mediated coupling (75%) and finally alane reduction (72%) gave the target compound. Related compounds were similarly obtained in excellent yields. The determinants for high sigma receptor affinity of 1 were examined by manipulation of the structure in a number of ways. The effect of changing the N-alkyl substituent of 1 indicated that the N-Me group (i.e., 1 itself) is the optimal N-substituent. This study also indicated that the boundary condition for high sigma receptor affinity occurs at N-propyl, since the affinity of the N-butyl homolog drops off 10-fold (Ki=11.7 nM compared with Ki=1.35 nM for the N-propyl compound). Opening up of the pyrrolidinyl ring of 1 as in the N,N-dimethyl and N,N-diethyl compounds, resulted in lowered affinity indicating that an intact pyrrolidine ring is important for high sigma receptor affinity. Examination of the effect of either lengthening or shortening of the distance between the N-methyl nitrogen and pyrrolidine as well as N-methyl nitrogen and 3,4-dichlorophenyl ring indicated that an ethylene moiety is optimal for both distances. Examination of the aromatic ring substituents revealed that the deschloro compound N-[2-(phenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine is also a potent sigma receptor ligand (Ki=7.40 nM) but is 22-fold less potent than 1. This suggests that 3,4-dichloro substituents are important for high affinity. The 3- and 4-monochloro analogs are both more potent than the deschloro compound but are less potent than 1 (Ki=0.84 nM for meta chloro; Ki=1.47 nM for para). Examination of the effect of ring size revealed that the potency of 1 for the sigma receptor could be still further improved by substitution of the 5-membered ring of 1 with a six and seven membered ring. These ring enlarged homologs displayed Ki values of 0.26 nM and 0.17 nM, respectively. In parallel assays with these compounds, $[^3H]$-(+)-3-PPP exhibited an Kd of 27.4 nM. Of the 22 compounds in this series tested, 16 of them exhibited a Ki of less than 2 nM and 8 of them showed subnanomolar potency. The high efficacy of these compounds for the sigma receptor, their relative chemical simplicity and ease of synthesis compared with other commercially available sigma ligands and their high degree of selectivity makes N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine and related compounds a highly promising base for selection of probes for determination of the functional role of sigma receptors as well as for the development of novel therapeutic agents.

Synthesis and Evaluation of N-Substituted cis-N-Methyl-2-(1-pyrrolidinyl)-cyclohexyl amines as High Affinity Sigma Receptor Ligands. Identification of a New Class of Highly Potent and Selective Sigma Receptor Probes. Certain benzeneacetamides [(-)- and (+)-cis-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexylbenzeneacetamide] were recently reported to be potent sigma receptor ligands. In order to determine whether efficacy for the sigma receptor could be improved, a series of compounds related to them, N-substituted cis-2-(1-pyrrolidinyl)-N-methylcyclohexylamines, were synthesized. The synthesis started with the previously reported racemic,1S,2R-(+)-, and IR,2S(-)-cis-2-(l-pyrrolidinyl)-N-methylcyclohexy-amines. Analysis of sigma ([3H](+)-3-PPP), kappa ([3H]-bremazocine and [3H]-U69,593), dopamine-d2 ([3H](-)-sulpiride), and PCP ([3H]TCP) receptor binding in guinea pig brain revealed a number of highly potent and selective sigma receptor ligands. Notably, 1S,2R-cis-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-(2-naphthyl)acetamide (Ki=8.66), racemic cis-2-amino-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (Ki=3 nM), 1S,2R-(-)-cis- and IR,2S-(+)-cis-N-methy-N-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexy-lamine (Ki=1.3 and 6 nM, respectively) exhibited very high affinity at sigma receptors, by displacement of [3H](+)-3-PPP. These compounds showed insignificant affinity for kappa, dopamine, or PCP receptors, making them valuable tools for the study of sigma receptors. Furthermore, these compounds also exhibited enantioselectivity ranging from 5-fold to 160-fold. Several other compounds showed equivalent selectivity but displayed lower sigma receptor affinity.

Synthesis and Characterization of Optically Pure [3H](+)-Azidophenazocine ([3H](+)-AZPH), a Novel Photoaffinity Label for Sigma Receptors. [3H](+)-cis-N-(2-(4-Azidophenyl)ethyl)-2'-hydroxy-2,6-dimethyl-6,7-benzomorphan ([3H](+)-AZPH), a novel high affinity and high selectivity benzomorphan based photoaffinity label for sigma receptors was synthesized in 4 steps starting with optically pure (+)-normetazocine and 2-(p-azidophenyl)ethyl methanesulfonate ester. Condensation of these compounds in DMF in the presence of NaHCO3 afforded (+)-AZPH in 77% yield. The tritiation precursor (+)-cis-N-(2-(4-azidophenyl)ethyl)-2'-hydroxy-1',3'-dibromo-2,6-dimethyl-6,7-benzomorphan for medium specific activity [3H](+)-AZPH was obtained in 96% yield by bromination of (+)-AZPH with 2 equivalents of bromine. The sequence of catalytic tritiation of the precursor, treatment of the resulting aniline (13.5% radiochemical yield, specific activity 22.9 Ci/mmol) with nitrous acid followed by sodium azide afforded the target compound in 78% chemical yield.

Wash-Resistant Inhibition of Phencyclidine and Haloperidol-Sensitive Sigma Binding Sites in Guinea Pig Brain by Putative Affinity Ligands: Determination of Selectivity. Several putative affinity ligands, based on the structures of etoxadrol, 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cycloheptene-5,10-imine (MK801), and

1,3-di-(2-methylphenyl)guanidine (DTG) were evaluated in vitro for their ability to produce a wash-resistant inhibition of PCP and sigma sites in homogenates of the brain of the guinea pig. All the PCP-based ligands, including 1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine (Metaphit) and a racemic MK801-NCS, produced a wash-resistant inhibition of binding sites, for phencyclidine labelled by [3H]-1-[1-(2-thienyl)cyclohexyl]piperidine [3H]TCP) and sigma binding sites, labelled by [3H]DTG. The DTG-based ligands, 1-(4-isothiocyanato-2-methylphenyl)-3-(2-methylphenyl)guanidine (DIGIT) and 1-{4-[2-(2-isothiocyanatoethoxy)ethoxy]-2-methylphenyl}-3-(2-methylphenyl)guanidine (DIGIE), produced a wash-resistant inhibition of sigma sites, at concentrations as small as 1 micromolar and also inhibited binding sites for phencyclidine at larger concentrations (100 micromolar). Both etoxadrol-NCS and 1-[1-(3-bromoacetyloxyphenyl)cyclohexyl]-1,2,3,6-tetrahydropyridine (Bromacetyl-PCP) were the most potent and selective inhibitors of the binding of [3H]TCP, while DIGIT was the most selective inhibitor of the binding of [3H]DTG. Future studies will examine the selectivity of these agents in vivo after intracerebroventricular administration.

Labeling by [3H]1,3-Di(2-tolyl)guanidine of Two High Affinity Binding Sites in Guinea Pig Brain: Evidence For Allosteric Regulation by Calcium Channel Antagonists and Pseudoallosteric Modulation by Sigma Ligands. Equilibrium binding studies with the sigma receptor ligand [3H]1,3-di(2-tolyl)guanidine ([3H]DTG) demonstrated two high affinity binding sites in membranes prepared from guinea pig brain. The apparent Kd values of DTG for sites 1 and 2 were 11.9 and 37.6 nM, respectively. The corresponding Bmax values were 1045 and 1423 fmol/mg of protein. Site 1 had high affinity for (+)-pentazocine, haloperidol, (3)-(+)-PPP, carbetapentane, and other sigma ligands, suggesting a similarity with the dextromethorphan/sigma-1 binding site previously described by other workers. Site 2 had high affinity for DTG and haloperidol (Ki=36.1 nM) and low affinity for most other sigma ligands. Kinetic experiments demonstrated that [3H]DTG dissociated in a biphasic manner from both site 1 and site 2. DTG and haloperidol increased the dissociation rate of [3H]DTG from site 1 and site 2, demonstrating the presence of pseudoallosteric interactions. Inorganic calcium channel blockers such as Cd2+ selectively increased the dissociation rate of [3H]DTG from site 2, suggesting an association of this binding site with calcium channels.

The Competitive NMDA Receptor Antagonist, CPP, Allosterically Modulates NMDA Receptor Associated PCP Binding Sites. We have determined that the competitive NMDA receptor antagonist, CPP, allosterically modulates NMDA receptor associated PCP binding sites in the absence of steric hindrance. In our experiment, equilibrium binding conditions were achieved within 2 h for [3H]TCP with both prep 1 and prep 2. CPP inhibited the binding of [3H]TCP with both membrane preparations; this inhibition was observed at equilibrium. The inhibition of binding resulted in a lengthening in the time required to reach equilibrium such that 6 h was required for [3H]TCP in the presence of CPP. The inhibition produced by CPP resulted from an 1.5-fold increase in the Kd of TCP for PCP receptors; the inhibition of binding by CPP did not effect the Bmax. The inhibition of [3H]TCP binding produced by CPP was completely reversed by glutamate. Glutamate, itself, had no effect on [3H]TCP binding. The initial rate of association of [3H]TCP increased 7.0-fold (prep 1) and 7.4-fold (prep 2) in the absence of CPP and 11.1-fold (prep 1) and 8.1-fold (prep 2) in the presence of CPP with a 10 fold increase in the TCP concentration. Similar results were obtained with the [3H]MK801 binding assays, although quantitative differences were observed. Thus, our experimentally determined association time course studies suggest that CPP allosterically modulates NMDA-associated PCP binding sites in the absence of steric hindrance since the inhibition of binding produced by CPP persisted at equilibrium and was reversed by glutamate and the initial rate of association of PCP ligands in the presence of CPP increased proportionally to their concentration.

BIOMEDICAL CHEMISTRY SECTION

Studies Related to the Human Immunodeficiency Virus

Facile Preparation of 32-P Labelled AZT Triphosphate. A quick and simple method has been devised to permit the microscale synthesis of [alpha-32P]-AZT which is valuable as a probe of the mechanism of action of AZT. This approach may also be useful for application to related dideoxynucleoside inhibitors of HIV. By the combination of a variety of synthetic techniques, a novel analogue of AZT 5'-triphosphate has been prepared; namely, the beta, gamma-difluoromethylenephosphonate analogue This material possesses a beta-gamma bond that cannot be hydrolyzed, and its pKa is similar to the parent AZT triphosphate. This analogue was found to be 30-fold less potent as a HIV reverse transcriptase inhibitor than AZT triphosphate, but it was 10 times more effective than the corresponding phosphonate analogue without the fluoro substitution, suggesting that this substitution provides a closer match to the oxygen bridge of the parent compound, AZT triphosphate.

Conformation of an HIV Reverse Transcriptase Inhibitor. The recent finding that 3'-amino-3'-deoxythymidine 5'-triphosphate is a noncompetitive inhibitor of the HIV-I reverse transcriptase, prompted an investigation of the conformation of 3'-amino-3'-deoxythymidine. An x-ray diffraction study has revealed that the glycosidic torsion angle of the nucleoside is in the less common syn-region and this solid-state geometry is stabilized by a three-dimensional network of self-associated hydrogen-bonded molecules. On the other hand, the aqueous solution conformation, as determined by IH-NMR, places the glycosidic torsion angle in the more usual anti-region with the sugar in an equilibrium between C3'-endo and C2'-endo puckering The energy barrier between the solid-state and solution conformation is relatively low as was demonstrated by the MM2-calculations.

CARBOHYDRATES SECTION

Reactions and Immunochemistry of Carbohydrates

Physico-chemical Studies on Antigen-Antibody Systems. The equilibrium binding constants (Ka) of four different monoclonal antibodies with mono, di-, tri-, tetra-, penta-, and hexa-oligosaccharides were measured at temperatures of 5, 13, 25, and 45C. The method employed was based on measurements of the intrinsic fluorescence changes of the protein upon binding to the saccharidic ligands. Two of the antibodies studied were groove-binding: antigalactan myelomas IgA X24 and IgA J539. The other two were cavity-binding: antidextran myeloma IgA W3129 and hybridoma IgA 16.4.12E. The binding pattern of IgA X24 and IgA J539 with methyl b-(1,6)-linked oligogalactosides is similar. They bind monosaccharides at 5C with Ka of 1000 L/M and 2600 L/M, respectively and hexasaccharide at 5C with Ka of 1,500,000 L/M and 9,500,000 L/M, respectively. Similarly, the myeloma IgA W3129 and hybridoma IgA 16.4.12E bind alpha-methyl glucoside at 5C with Ka of 4500 L/M and 10,000 L/M, respectively and methyl alpha-(1,6)-linked hexaglucoside at 5C with Ka of 350,000 L/M and

1,600,000 L/M, respectively. Comparison of Ka from mono- to hexasaccharides for both antibodies indicates that four saccharide units are needed for optimal binding (site saturation) at any temperature from 5C to 45C. The binding constants for all four antibodies and oligosaccharides studied decreased with increasing temperature. The free energy of binding of each antibody with its ligands at given temperature can be calculated from equation: dG = -RT ln Ka. The calculated values of free energies of binding for each of the four antibodies with its ligands at different antibodies are negative, from -3 to -9 kcal. It can also be seen that the monosaccharide contributes about 40% of total binding energy in case of the antigalactan groove-binding IgA X24, whereas this value is increased to ca. 60% for the antidextran cavity-binding IgA 16.4.12E antibody. The application of Van 't Hoff equation (-RT ln Ka = dH - TdS) allows for calculation of thermodynamic parameters, enthalpy and entropy. All studied antibodies and their ligands show negative entropy, which is compensated for by a profitable energetic factor due to high negative enthalpy. The comparison of thermodynamic parameters for the antigalactan groove-binding versus antidextran cavity-binding antibody shows that enthalpy and entropy changes in the first case are significantly smaller for monosaccharide than for higher oligosaccharides, whereas in the second case these values are comparable for all ligands studied. Thus, for in the groove binders the counter-productive (to binding) dS increases with increasing oligosaccharide size, perhaps indicating immobilization on the antibody surface, while in the case of the cavity binders the initial, counter-productive entropy change does not increase much for the larger oligosaccharidic determinants, indicating that most of the binding takes place to the terminal glucosyl group. (Drs. E. Nashed and C. Glaudemans)

Oligosaccharides were constructed that contained part or all of the immunodeterminant of the anti-galactan antibodies flanked by non-specific (that is non-binding) carbohydrate residues. In that way the capability of the antibody to read homologous determinants "hidden" among unrelated structures could be evaluated. Oligosaccharides were constructed having beta-galactopyranoside, 1,6-linked beta-galactopyranosyl di-, tri- and tetra-saccharides flanked (on both sides) by beta-1,6-linked-gentiobioside. Measurements of the affinity constants with anti-galactan antibody showed that the antibody could read even a single galactose residue in the flanked pentasaccharide and can thus read internal immuno-determinants. (Drs. Th. Ziegler and C. Glaudemans)

Studies on the Immunodeterminant of Shigella dysenteriae Type 1. The determination of the size of the binding site of an immunoglobulin and the investigation of the nature of forces involved in the binding process requires a large number of ligands. In the case of carbohydrate specific antibodies, ligands constitute carbohydrate derivatives, mono and oligosaccharides, which are invariably obtained by chemical syntheses. In addition to mono and oligosaccharides related to the natural antigen, we also synthesize modified ligands. To those belong molecules having, for example, certain hydroxyl groups replaced by functions which modify the binding pattern, thus allowing us to draw conclusions about the importance of various forces in the binding process. During our previous studies with systems which involved carbohydrate homopolymers we have developed a rational process for obtaining detailed information, on the molecular level, on the mode of binding. As a logical next step we want to apply this proven approach and methods in a more complex system, such as one involving an antibody specific to a polysaccharide antigen of which the determinant epitope is a repeating unit made up of several different sugar moieties. As the objective of our present investigation we have chosen to study the system comprised of the O-specific side chain of Shigella dysenteriae type 1 and its homologous antibody. From the found mode of interaction of ligands representing fragments of the chemical repeating unit of the polymeric antigen, as well as their deoxygenated and fluorinated analogs, with the respective antibodies, we expect to be able to draw conclusions about the binding process. This may be helpful in the developing of a synthetic vaccine conceivably more powerful than the one based on the natural polymeric antigen. The parent bacterium is responsible for many deaths in infants and small children. Therefore, the development of a powerful vaccine which would control this devastating disease is well justified.

Methyl alpha-glycosides of oligosaccharides related to the chemical repeating unit of the O-polysaccharide of Shigella dysenteriae type 1 bacterial lipopolysaccharide:

-[3-alpha-L-Rha(1,3)-alpha-L-Rha(1,2)-alpha-D-Gal(1,3)-alpha-D-GlcNAc-
1]n-
A B C D

having N-acetylglucosamine as the glycone-bearing terminus have been synthesized. The extension of the oligosaccharide chain, to obtain target fragments DA, DAB and DABC, was achieved by a stepwise approach. Condensations aimed at the formation of 1,2-cis-glycosidic linkage, such as in sequences DA, DAB and DABC, were performed under the conditions of silver perchlorate-mediated glycosylations using a glycosyl donor having a non-participating group at C-2. Condensations targeted towards intermediates containing 1,2-trans-glycosidic linkages, such as in sequences AB and ABC, were performed with 1,2-trans-directing glycosyl halides under the conditions of base-deficient silver triflate-mediated glycosylation reactions. The construction of the three oligosaccharides containing the alpha-D-glucosamine as the glycon-bearing terminus by the stepwise approach required the construction of the alpha-D-glycosidic linkage of glucosamine. In the 2-aminosugar series this is most effectively accomplished using 2-azido-2-deoxy-glycosyl donors. The glycosyl donor used for this purpose was 1,3,4,6-tetra-O-acetyl-2-azido-2-deoxy-alpha-D-glucopyranosyl chloride, for which we developed an efficient synthesis starting from D-mannose. D-Mannose is first converted to 1,3,4,6-tetra-O-acetyl-beta-D-mannopyranose and subsequently trifluoro-methanesulfonylated. The 2-trifluoromethanesulfonyl group is then displaced with the azido ion to form the corresponding 2-azido-2-deoxy derivative having the D-gluco configuration. Treatment of the latter with 1,1-dichloromethyl methyl ether affords the target glycosyl donor, 1,3,4,6-tetra-O-acetyl-2-azido-2-deoxy-beta-D-glucopyranosyl chloride. To make the methyl alpha-glycoside of the disaccharide DA this glycosyl chloride was condensed with the 2,4-di-O-benzoyl derivative of methyl alpha-L-rhamnopyranoside, and the fully protected disaccharide was deacylated.

The construction of the higher oligosaccharides in this series required the preparation of a complex glycosyl donor derived from L-rhamnose, having a selectively removable protecting group at O-3. We have chosen for this purpose the bromoacetyl group which can be selectively removed in the presence of other acyl groups. Methyl 2,4-di-O-benzoyl-3-O-bromoacetyl-alpha-L-rhamnopyranoside was prepared according to the technology previously developed in our laboratory. The crystalline product obtained was cleaved with dichloromethyl methyl ether in the presence of zinc chloride and the glycosyl chloride thus obtained was condensed with methyl 2,4-di-O-benzoyl-alpha-L-rhamnopyranoside. The proton coupled 13C-nmr spectrum of the formed disaccharide contained two doublets at delta 99.2 and 98.4, showing JC-1,H-1 171.5 and 170.8 Hz, respectively, characteristic of a glycosidic linkage. The fully protected disaccharide was de-O-bromoacetylated with thiourea yielding a disaccharide nucleophile, which was subsequently condensed with 1,3,4,6-tetra-O-acetyl-2-azido-2-deoxy-beta-D-glucopyranosyl chloride. The resulting, fully protected trisaccharide was deacylated, to give the methyl alpha-glycoside of the trisaccharide DAB. To construct the sequence DABC, the complex glycosyl donor, 2,4-di-O-benzoyl-3-O-bromoacetyl-alpha-L-rhamnopyranosyl chloride, was first condensed with methyl 3-O-benzoyl-4,6-O-benzylidene-alpha-D-galactopyranoside. The resulting disaccharide was debromoacetylated, and the resulting disaccharide nucleophile was condensed again with the same rhamnosyl glycosyl donor. After the debromoacetylation of the fully protected trisaccharide obtained, the resulting trisaccharide nucleophile was condensed with 1,3,4,6-tetra-O-acetyl-2-azido-2-deoxy-beta-D-glucopyranosyl chloride. Deacylation of the fully protected tetrasaccharide gave then the target methyl glycoside. Its proton-coupled 13C-nmr spectrum showed four doublets showing splittings of 173.0, 170.7, 173.9, and 171.2, confirming that all glycosidic linkages were alpha. (Drs. P. Kovac, V. Pavliak and C. Glaudemans)

The tri- to hexa-saccharides listed below from the above immunodeterminant sequence were also synthesized: CDA, BCDA, CDAB, ABCDA and ABCDAB. In the process a new, stereo-specific, 1,2-cis migration in thioglycosides was discovered: when the the thio-alpha-methyl glycoside of 4-O-benzoyl-L-rhamnopyranoside was treated for an extended time with trimethyl-orthobenzoate-camphor sulfonic acid, the methyl-beta-glycoside of 2-S-methyl,3,4-di-O-benzoyl-L-rhamnopyranoside was obtained. The mechanism of this new reaction could be rationalized (Dr. V. Pozsgay).

Studies on the Binding Patterns of All Synthetic Immunodeterminants to Murine Monoclonal Anti-Shigella dysenteriae Type 1. In order to define the antigenic determinant (epitope) of Shigella dysenteriae type 1 LPS, interactions of the IgM were evaluated with all the above methyl alpha-glycosides of mono- and oligosaccharides representing a part or the whole repeating unit of O-antigen. The results obtained show that the subsite having the affinity for the galactosyl residue is likely to be near a solvent-exposed tryptophanyl residue. Extension of the oligosaccharide chain towards the nonreducing end of the O-antigen shows increased perturbation indicating that the L-rhamnopyranose (B) attached to alpha-D-galactopyranose (C) can either interact with the solvent exposed tryptophanyl residue located near the galactosyl residue-binding site (thus causing increased perturbation), or there is a second solvent exposed tryptophanyl residue near that L-rhamnosyl residue binding subsite.

The lower affinity observed for alpha-glycosides of CD, BCD and ABCD, compared with those for C, BC and ABC indicates that the N-acetylglucosamine at the aglyconic terminus of the ligand might cause a conformational change decreasing optimal binding in the antibody's binding area. The conformation becomes more optimal for binding when ligands have their chain extended from the N-acetylglucosamine unit towards the reducing end of the O-antigen (CDA, CDAB, BCDA, ABCDA).

Shifting of the tetrasaccharides along the antibody's binding site shown by different affinities in the order of CDAB<BCDA<ABCD<DABC suggests that the tetrasaccharide dcba represents the possible epitope of Shigella dysenteriae type 1 O-antigen. The individual affinity constants for the subsites of antibody's binding area and the corresponding sugar units of the tetrameric epitope, calculated using the additivity of the free energies of binding, are 180 L/M for C, 40 for B, 1.1 for A, and 1.3 for D. (Drs. V. Pavliak and C. Glaudemans)

Studies on Monoclonal Antidextran Antibodies. Methyl O-(6-O-acetyl-2,3,4-tri-O-benzyl-alpha-D-glucopyranosyl)-(1,6)-2,3,4-tri-O-benzyl-alpha-D-glucopyranoside was treated with acetic acid, acetic anhydride and sulfuric acid to give 1-O-acetyl-O-(6-O-acetyl-2,3,4-tri-O-benzyl-alpha-D-glucopyranosyl)-(1,6)-2,3,4-tri-O-benzyl-alpha-D-glucopyranose in 81% yield. After reaction with ethereal hydrogen chloride, a 76% yield was obtained, and this was used for glycosylation reactions with the appropriate nucleophile to give methyl O-alpha-D-glucopyranosyl-(1,6)-alpha-D-glucopyranosyl-(1,6)-2-deoxy-, 3-deoxy and 4-deoxy-alpha-D-glucopyranosides. These oligosaccharides possess deoxy functions, and thus allow them to be used in the evaluation of the role of hydrogen bonding between dextran-like epitopes and monoclonal antibodies. (Drs. E. Petrakova and C. Glaudemans)

Sequence of the VH Gene of Murine IgA X-24. Straight forward cloning of the VH gene, including the flanking region necessary for expression was not possible due to the clustering of a number of commonly used restriction sites within, and adjacent to, the coding region. To overcome this problem, two clones were isolated from an EMBL-3 library prepared from X-24 DNA. The first begins with the BAM H1 site at nucleotide 587 of the rearranged gene, and contains the coding region from amino acid 17 to the end of the VH. The second is a germline clone, also beginning at nucleotide 587, but extending 4.3 kb in the 5 prime direction, thus containing the first 17 amino acids of the coding region as well as regulatory sequences necessary for expression. Appropriate fragments were prepared from these two clones, ligated and subcloned to yield a construct containing the 5-prime region and first 46 nucleotides of the coding region from the germ line gene encoding X-24 VH with the remainder of the V region derived from the rearranged gene. Sequence analysis revealed a leader of 18 amino acids, consenses splice sites, 98 amino acids in the VH, and 12 in the joining segments. Conserved sequence elements, including heptamer motif, octamer motif and pyrimidine-rich area necessary for appropriate expression are also found in the 5-prime region. It was noted that two heptamer motifs, both preceded by pyrimidine rich stretches were observed, rather than the the one normally found in other VH genes.

Attempts continue to find proper expression for our cloned immunoglobulin genes. A construct containing both the VH and the VL has been made. Expression of this construct in E. coli is hindered by poor growth of the vector, possibly due to toxicity of the VH.(Drs. Rao Arepalli and C. Glaudemans)

1990-1991 Non-project Activity.

Dr. Kenner C. Rice continues as a member of the Editorial Advisory Board of the Journal of Medicinal Chemistry and has completed his three year term as an elected member of the Executive Committee of the Organic Chemistry Division of the American Chemical Society (ACS). In the latter capacity, he served as liaison with the Biotechnology Secretariat of the ACS. He continues as an elected member of the Board of the Committee of Problems on Drug Dependence (CPDD) representing the American Chemical Society. Dr. Rice served as a member of Peer Review Panel C for the Walter Reed Army Institute of Research Promotion Board. During the reporting period, Dr. Rice has been invited to present a number of research lectures in the US and abroad. These include presentations in symposia on drugs of abuse as a Keynote Speaker at the 1992 American Society of Pharmacognosy meeting and at the 1992 meeting of the American Association of Pharmaceutical Scientists. Dr. Rice was reappointed as Adjunct Professor of Pharmacology in the Department of Pharmacology and Experimental Therapeutics, School of Medicine, University of Maryland, Baltimore. He continues to serve on the selection committee for national and international research awards.

Dr. Arthur E. Jacobson was reappointed as Biological Coordinator of the Drug Evaluation Committee of the College on Problems of Drug Dependence for 1991-1992, and as Affiliate Professor in the Department of Pharmacology and Toxicology, Medical

College of Virginia, Virginia Commonwealth University. He has been appointed to the Editorial Board of the international journal Drug and Alcohol Dependence which publishes work on biomedical and psychosocial approaches in these areas of dependence. He continues to serve on the selection committee for the international Sato Memorial award, and is on the Research Evaluation Panel for the Walter Reed Army Institute of Research. He has been invited to serve as Chairman for the Chemistry and Molecular Modeling/Molecular Biology session, and lecture at the Third French-U. S. Joint Symposium on Multiple Sigma and PCP Receptor Ligands, in Le Grande Motte, France.

Dr. Richard B. Rothman was selected as the 1991 recipient of the Joseph Cochin Young Investigator Award by the Committee on Problems of Drug Dependence (CPDD). The CPDD is the nation's preeminent scientific society for research in all disciplines of substance abuse research. The award recognized Dr. Rothman's research contributions in the area of opioid pharmacology.

Dr. Cornelis P. J. Glaudemans served as a member of the Editorial Board of the international journal Carbohydrate Research. In the past year, he served as Chairman of the Gordon Conference on Carbohydrates. He has been invited as a plenary speaker at conferences in Japan, Scotland and Czechoslovakia.

Dr. Paul Kovac served as a member of the Editorial Board of the international journal Journal of Carbohydrate Chemistry. He has been invited as a plenary speaker at the International Carbohydrate Symposium in Japan and at the Gordon Conference on carbohydrates in Tilton, NH.

Patent Applications filed during 1990-1991. In the Drug Design and Synthesis Section, LMC, four new patents were filed, and one patent was issued. These inventions include (a) Nitrogen-containing cyclohetero cycloalkylaminoaryl derivatives for CNS disorders; (b) N-(arylethyl)-N-alkyl-2-(1-pyrrolidinyl)ethylamines, a novel class of neuroprotective sigma receptor ligands; (c) radiolabeled N-substituted-6-iodo-3,14-dihydroxy-4,5-alpha-epoxymorphinans, intermediates for producing the same, and a process for the preparation and methods of detecting opioid receptors; and, (d) (+)-isomers of endoetheno/endoethano-epoxymorphinan derivatives as antitussive agents. The issued patent concerned: Method of synthesis of hydroxy-substituted 4-alkoxyphenylacetic acids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 59,501-05 LMC

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Design and Synthesis of Drugs Acting on Central.and Peripheral Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: K. C. Rice, Laboratory Chief, LMC-NIDDK

Co PI: A. E. Jacobson, Deputy Chief, LMC-NIDDK

OTHERS:

B.R. de Costa, Visiting Associate; N.A. Grayson, IRTA; M. Seggel, IRTA; R.J. Weber, Staff Fellow; M. Mattson, Biologist; L. Band, IRTA; W. Williams, Microbiologist; H. Xu, Visiting Associate; R.B. Rothman, Senior Research Investigator; H. Akunne, Fogarty Fellow; L. Radesca; K. Yamada, Special Volunteer; J.T.M. Linders, Visiting Fellow; D. Tadic, Visiting Fellow. All LMC, NIDDK.

COOPERATING UNITS *(if any)*

LN-NIDDK (P. Skolnick); U Arizona (F. Porreca, Q. Jiang); U of Michigan (J. Woods, C. P. France, F. Medzihradsky, C. B. Smith, G. Winger); CC-NM (D. Kiesewetter, M. Channing); U Alabama (E. Blalock); BPB-NIMH (A. Pert); Research Triangle Institute (F. I. Carroll); ARC, NIDA (J. L. Katz).

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Drug Design and Synthesis Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 11.0	PROFESSIONAL: 9.5	OTHER: 1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The primary goals of this program are to gain further insight into the structure and function of neurotransmitter systems in the overall operation of the mammalian central nervous system (CNS), and the molecular mechanism of action of drugs which act on the CNS, as well as the mechanism(s) through which the immune and other peripheral systems are influenced by the CNS in normal and disease states. The multidisciplinary approach utilized in this program employs rational drug design based on structure-activity relations and molecular modeling, modern organic chemical synthesis, pharmacology, biochemistry, immunology and requires collaboration with other appropriate disciplines. Elucidation of the molecular structure and mechanism of action of the ligand-receptor systems and their antagonists provides new opportunities for the design of superior drugs and understanding disorders for which the pathogenesis is largely obscure. Synthetic programs are continuing to develop new ligands for imaging brain drug receptors by positron emission tomography (PET) and single photon emission computed tomography (SPECT) scanning. The NIH Opiate Total Synthesis continues to be employed to provide previously inaccessible unnatural enantiomers of opiates and derivatives (e.g., (+)-buprenorphine and (+)-diprenorphine) as new pharmacological agents and research tools. (+)-Buprenorphine is a potentially valuable research tool in mechanistic studies of suppression of cocaine self-administration by (-)-buprenorphine. The role of classical opiate receptors in cocaine addiction is now being intensely studied. (-)-Buprenorphine appears to protect against the lethal effects of cocaine by a process mediated by mu-opioid receptors

Oxide-bridged 5-phenylmorphans are being studied, through synthesis and computer-assisted molecular modeling, as probes for narcotic receptor mediated phenomena. Irreversible antagonists to selective opioid receptors are also under investigation. Our recently synthesized UPHIT has been found to irreversibly inhibit binding at kappa sites and may prove useful in determining the biological function of kappa receptor subtypes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 59,502-05 LMC

PERIOD COVERED
October 1, 1990 through September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Design, Synthesis and Evaluation of Medicinal Agents and Research Tools

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: A. E. Jacobson, Deputy Chief, LMC-NIDDK
Co PI: K. C. Rice, Laboratory Chief, LMC-NIDDK
OTHERS:
A. A. Reid, Biochemist, LMC-NIDDK; B. R. de Costa, Visiting Associate, LMC-NIDDK; P. Hillery, Special Volunteer, LMC-NIDDK; M. Mattson, Biologist, LMC-NIDDK; H. Akunne, Fogarty Fellow, LMC-NIDDK; L. Radesca, Special Volunteer, LMC-NIDDK; J. T. M. Linders, Fogarty Fellow, LMC-NIDDK; S. Richardson, IRTA, LMC-NIDDK; R. B. Rothman, Sr. Research Investigator, LMC-NIDDK; N. A. Grayson, IRTA, LMC-NIDDK; R. J. Weber, Staff Fellow, LMC-NIDDK; H. Xu, Visiting Associate, LMC-NIDDK.

COOPERATING UNITS *(if any)*
Brown University, (W. Bowen, D. T. Finn, S. Vazirani); WRAIR (J. B. Long); U of Michigan (J. H. Woods, C. P. France); MN-NINDS (M. A. Rogawski); BPB-NIMH (A. Pert); Medicis (J. W. Holaday); Searle-Monsanto (P. C. Contreras, N. M. Gray, I. Daly, S. Iyengar).

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION
Drug Design and Synthesis Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	5.0	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies are in progress towards the design and synthesis of new phencyclidine (PCP)-like compounds as neuroprotective agents and as anticonvulsants. PCP-like compounds have been reported to exert a protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of N-methyl-D-aspartate (NMDA) in animal brain. PCP binding sites exist in excitatory amino acid controlled ion channels regulated by glutamate receptors of the NMDA type, as well as in the dopamine uptake complex. Our demonstration of the existence of a high affinity PCP binding site associated with the dopamine reuptake carrier raises the possibility that the therapeutic and psychotomimetic effects of PCP in humans are separable and mediated via different binding sites. We also synthesized two MK-801 isothiocyanates as potential affinity ligands. One of these has proven to be more potent and selective in its interaction with the PCP binding sites than our former affinity ligands; it interacts minimally with the dopaminergic reuptake system.

Sigma receptors are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs and have been implicated in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. We have identified a novel class of high-affinity sigma receptor ligands, the enantiomeric N-substituted cis-N-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl)cyclohexylamines. The most potent, site selective, sigma ligand found in this group was the 1R,2S-(-)-unsubstituted derivative (Ki=0.49 nM). Related compounds constitute a new class of superpotent sigma ligands. Optically pure [3H](+)-cis-N-(2-(4-azidophenyl) ethyl)-2'-hydroxy-2,6-dimethyl-6,7-benzomorphan was synthesized and characterized as a high affinity and highly selective benzomorphan-based photoaffinity label for sigma receptors.

Cannabinoid receptors were characterized and localized through an in vivo autoradiographic study in rat brain. Neuronal localization of these receptors occurred in the basal ganglia of the rat. The function of these receptors in the brain is being sought.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 59601-04 LMC
formerly -
Z01 58014-03 LAC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.F. Torrence	Research Chemist	LMC/NIDDK
Other:	K. Lesiak	Visiting Scientist	LMC/NIDDK
	T. Kovacs	Visiting Fellow	LMC/NIDDK
	S. Khamnei	Visiting Fellow	LMC/NIDDK

COOPERATING UNITS *(if any)* FOREIGN: Rega Institute, Catholic Univ. of Leuven, Belgium (Dr. E. DeClercq and J. Balzarini); Nagoya City Univ., Japan (Dr. K Kohda). NIH: Dr. N. Greig, NIMH; Dr. S. Wilson, NCI. Dr. K. Pannell (Univ. Texas, El Paso), Dr. I. Pelczer (Univ. Syracuse); Dr. K. Kirk, NIDDK; Dr. D. Hebel, NIDDK.

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION
Biomedical Chemistry

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A quick and simple method has been devised to permit the microscale synthesis of $[\alpha\text{-}^{32}P]$-AZT which is valuable as a probe of the mechanism of action of AZT. This approach may also be useful for application to related dideoxynucleoside inhibitors of HIV. By the combination of a variety of synthetic techniques, a novel analogue of AZT 5'-triphosphate has been prepared; namely, the beta, gamma-difluoromethylenephosphonate analogue. This material possesses a beta-gamma bond that cannot be hydrolyzed, and its pK_a is similar to the parent AZT triphosphate. This analogue was found to be 30-fold less potent as an HIV reverse transcriptase inhibitor than AZT triphosphate, but it was 10 times more effective than the corresponding phosphonate analogue without the fluoro substitution, suggesting that this substitution provides a closer match than the oxygen bridge of the parent compound, AZT triphosphate.

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 59602-18 LMC
formerly -
Z01 58005-17 LAC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less Title must fit on one line between tha borders.)*
Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs-

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	P.F. Torrence	Research Chemist	LMC/NIDDK
Other:	K. Lesiak	Visiting Scientist	LMC/NIDDK
	T. Kovacs	Visiting Fellow	LMC/NIDDK
	W. Forkman	Visiting Associate	LMC/NIDDK

COOPERATING UNITS *(if any)*
R. Silverman, Dept. Pathology, USUHS, Bethesda, MD; P. Herdefiwn, Rega Institute, Leuven, Belgium.

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION
Biomedical Chemistry

INSTITUTE AND LOCATION
NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 3.5	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Interferon-induced enzyme activities such as the oligo (2'5') adenylate synthetase, and the oligo (2'5') A phosphodiesterase are investigated with the goal of understanding their role in the action of interferon, the induction of interferon by double-stranded RNA and, perhaps, control of cell growth and differentiation. Analogs of the mediator of interferon action, 2-5A, are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A dependent endonuclease. The eventual goal is to understand the biological role of the 2-5A system and to explore the potential of exploitation of this system in chemotherapy.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 59701 19LMC
Formerly
Z01 DK 19001 18LC

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Reactions and Immunochemistry of Carbohydrates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: Cornelis P. J. Glaudemans, Chief SOC

Others:	R. S. Arrepalli	Visiting Scientist
	P. Kováč	Research. Chemist
	E. M. Nashed	Visiting Scientist
	V. Pavliak	Visiting Fellow
	E. Petráková	Visiting Associate
	V. Pozsgay	Visiting Scientist

COOPERATING UNITS *(if any)*
J. B. Robbins, NICHD
R. Schneerson, NICHD
A. Bax, NIDDK
J. Lehmann, Freiburg University, Germany
E. A. Kabat, Columbia University
Th. Ziegler, Stuttgart, Germany
Y. Tsumuraya, Japan

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION
Section on Carbohydrates

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD, 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	7	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Section works on the interaction of (complex) carbohydrate determinants with monoclonal antibodies (MAbs). The elucidation of this interaction - in great molecular detail - is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. We are executing:

1. Physico-chemical studies on antibody/antigen systems.
2. The synthesis of ligands for affinity studies.
3. The manipulation of immunoglobulin genes to produce specifically mutated genes expressing altered antibodies.
4. The study of immunodeterminants of bacteria causing significant diseases on a global scale, so as to evaluate procedures for vaccine development.

We have determined the specific interaction between microbial polysaccharides such as dextran and a number of monoclonal antibodies in the past. Continueing this work, we have measured the enthalpy and entropy of interaction for a number of anti-dextran and anti-galactan monoclonal antibodies.

We have prepared many complex fragments of the capsular polysaccharide of *Shigella dysenteriae* type 1 by sophisticated syntheses, and have mapped the binding area of a monoclonal antibody towards this disease-causing micro-organism. In this manner, the immuno-determinant of this polysaccharide could be defined.

We have studied the way in which immunoglobulins recognize specific determinants amidst non-specific flanking sequences.

PHS 6040 (Rev. 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—[illegible]

PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH: SUMMARY

PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH

Introduction:

The Branch conducts epidemiologic and clinical research relating to the origin, development and natural history of non-insulin-dependent diabetes and its complications, and obesity, particularly among the Pima Indian population of Arizona. The Branch is based in Phoenix, Arizona and the facilities include a field clinic located in the Hu Hu Kam Memorial Hospital at Sacaton, Arizona on the Gila River Indian Reservation and the clinical research center in the Phoenix Indian Medical Center in Phoenix, Arizona. The Branch consists of three sections, the Clinical Diabetes and Nutrition Section, which conducts clinical research and laboratory investigations, the Diabetes and Arthritis Epidemiology Section, which is involved primarily with the epidemiological and genetic studies in the Pima Indian population residing on the Gila River Indian Reservation and the Biometry and Data Management Section which provides data management and support services for the Branch. The Branch also serves as a WHO Collaborating Center in Diabetes assisting other units and collaborating centers in methodology, design and analysis of epidemiological and clinical research studies in non-insulin-dependent diabetes mellitus.

The Clinical Diabetes and Nutrition Section had continued to focus on understanding the determinants and pathogenesis of non-insulin-dependent diabetes and the determinants and pathogenesis of obesity. The longitudinal study of the metabolic characteristics that predict the development of non-insulin-dependent diabetes and the sequence of metabolic events that occur during its development has continued. This study continues to demonstrate that insulin resistance precedes the development of both impaired glucose tolerance and diabetes itself. Furthermore, studies have shown that insulin action aggregates in families and among those who have not yet developed diabetes demonstrates a trimodal frequency distribution. This observation is consistent with the hypothesis that a major determinant of insulin resistance is an autosomal gene with a codominant mode of inheritance. During the past year evidence has been derived from the same subjects which strongly suggests that there is genetic linkage between a marker on chromsome 4q and insulin resistance. Attempts are now underway to define the precise genetic locus responsible for this genetic linkage.

The biochemical basis of insulin resistance has continued to be a focus of studies within the section. Previously evidence of impaired glycogen synthase activity in subjects with insulin resistance has been documented. To investigate the basis for impaired activitation of glycogen synthase, biochemical studies of other enzymes in the activation pathway of glycogen synthase have been conducted. Evidence of a deficiency in phosphorylase phosphatase activity in insulin resistant subjects has been demonstrated, and possible reasons for this failure of activation are being pursued. Other avenues of investigation relating to deficiencies in insulin action have also been explored. Studies conducted by the section have convincingly demonstrated that tyrosine kinase is not the site of this deficiency. Normal activation of S6 kinase activity has been demonstrated and insulin resistant subjects have been shown to have normal casein kinase^2 activation by insulin. These findings suggest that abnormalities at the level of protein tyrosine phosphatase, which is rapidly suppressed in insulin sensitive subjects but not in insulin resistant subjects, might be the site of abnormalities responsible for insulin resistance. Consequently studies to characterize protein tyrosine phosphatase activity in skeletal muscle have been performed and the sequence of protein tyrosine phosphatase is being elucidated in an attempt to identify specific abnormalities that are associated with the failure to suppress activity in insulin resistant subjects. Similar studies are also being conducted with phosphorylase phosphatase.

Other clinical studies conducted by the section have investigated the relationship between insulin resistance and blood pressure and have shown that an interrelationship is not present in nondiabetic Pima Indians, whereas a strong relationship is present in Caucasians. This led to the hypothesis that abnormalities of the symphathetic nervous system may underlie this absence of relationship among the Pima. Studies of the possible mechanisms of the relationship between obesity and insulin resistance have been continued. Although the specific reasons for this relationship remain unclear, to new studies have been initiated to investigate possible interactions between obesity and membrane phospholipid concentrations as a possible explanation for this relationship.

A number of innovative approaches to the investigation of energy metabolism and its relationship to the development of obesity are being conducted. New findings suggest that the sympathetic nervous system is a determinant of metabolic rate in Caucasians but not in Pima Indians, suggesting that decreased sympathetic mediated energy expenditure may predispose to obesity among the Pima Indians. New studies on the relationship energy expenditure in the free-living state and obesity have been initiated using the doubled labelled water method to measure energy expenditure in free-living conditions.

The Diabetes and Arthritis Epidemiology Section has also continued its longitudinal studies of risk factors for diabetes and vascular complications in the total Pima Indian population residing on the Gila River Indian Reservation. Evidence that susceptibility to diabetes itself appears to be transmitted by a major autosomal gene has led to continuing efforts to identify such a gene by means of linkage analysis. During the past year many potentially informative families have been examined and lymphoblast cells immortalized to permit characterization of such families for genetic loci using restriction fragment length polymorphisms of known chromosomal location in order to permit linkage analysis to identify genetic loci that may be implicated in the causation of diabetes. Evidence that the susceptibility to diabetic nephropathy may be inherited independently of genetic determinants of diabetes mellitus has been strengthened by further studies of the occurrence of diabetic nephropathy in siblings. Families have been assembled to permit the collection of material for RFLP analysis for genetic linkage with diabetic nephropathy.

Evidence that insulin resistance is manifest in Pima Indian children at an early age has been reported and the investigation of factors relating to diabetic complications has been continued. Particular emphasis has been placed on the causes and development of diabetic nephropathy. Studies of the sequence of events that occur during the development of renal insufficiency have shown that a defect in glomerular pore size can be identified very early in the course of diabetes. Longitudinal studies will help determine whether this abnormality is responsible for the development of albuminuria and sequential studies of subjects with minimal elevations of albumin excretion will help to define factors that determine if such individuals will develop progressive renal insufficiency.

Collaborative studies with a number of other diabetes centers have continued. The Branch has been active in analyzing the results of the WHO Multinational Study of Vascular Disease in Diabetes. This study has demonstrated that the frequency of specific diabetic complications varies widely in diabetics from different centers and possible reasons for this variation have been proposed. Members of the branch have continued in both national and international activities as detailed in the individual section summaries.

Awards, Invited Lectures and other activites

Peter H. Bennett, M.D., F.R.C.P., F.F.C.M.

Indian Health Service, Special Director's Award for Excellence
for Outstanding Efforts in the Understanding and Treatment of Diabetes in American Indian and Alaska Natives.

Editorial Activities

Consultant Editor, Diabetes and Metabolism/Reviews
Consultant Editor, Acta Diabetologica
Associate Editor, Journal of Public Health Medicine
Associate Editor, American Journal of Epidemiology

Foreign Activities

Invited Speaker, Epidemiology Satellite, Pan American Congress of Endocrinology, Recife, Brazil, November, 1990

Member, WHO DIAMOND Study Advisory Board

Other Activities

Invited Speaker, National Diabetes Advisory Board, January 1991 and March 1991

Member, Arthritis Plan Task Force for National Institute of Arthrits and Musculoskeletal and Skin Diseases, January 1991

Member, Scientific Program Committee of the 14th International Diabetes Federation Congress

Chairman, Organizing Committee, Epidemiology Satellite, 14th International Diabetes Federation Congress, June 29 - July 1, 1991

Resident Faculty, World Health Organization/Japan-Diabetes Foundation Course on Epidemiological and Public Aspects of Diabetes Mellitus, Tokyo, Japan, August 21-29, 1991

Clinical Diabetes and Nutrition Section

The scientific mission of the section is to determine the etiology and pathogenesis of non-insulin dependent diabetes mellitus as it occurs among the Pima Indians of Arizona. Our current research efforts have therefore focused on a major prospective study of nondiabetic Pima Indians. From these studies, insulin resistance has been identified as a major risk factor for the development of the disease. We, therefore, are attempting to identify the mechanisms of insulin resistance in the population at the biochemical and molecular level. Obesity is also a risk factor for the development of the disease, possibly by worsening insulin resistance, and we are studying the causes of obesity and how obesity may worsen insulin resistance.

Cross-sectional and longitudinal study of the development of NIDDM

A cross-sectional and longitudinal study of a subset of the Pima Indian population began in 1982 with the specific aims of: 1) to identify the metabolic characteristic which is most predictive of the subsequent development of NIDDM among nondiabetic Pimas and 2) to document the sequence of metabolic events that occur during the transition from normal to impaired glucose tolerance and subsequently to marked fasting hyperglycemia and NIDDM. Approximately 300 subjects have been entered into this study and are restudied annually. A great deal of information has already been obtained from cross-sectional analyses of this data and approximately 40 individuals have now developed diabetes in the course of this long-term experiment. In parallel with the study of the Indian volunteers, we have also studied a large sample of age-matched Caucasians to determine which metabolic characteristics of the Indians are quite distinct from that of the Caucasians and therefore, might be the reason why the population has a higher prevalence of NIDDM.

Compared to Caucasians the Pima Indians are more insulin resistant on average. This is not only due to their greater prevalence of obesity, but is also independent of the effect of obesity per se. In addition, at the same degree of insulin resistance the Pimas secrete more insulin, particularly acutely after a stimulus such as ingestion of glucose or a mixed meal. These data suggest that the higher prevalence of diabetes in the Pima Indian population compared Caucasians is due to insulin resistance rather an abnormality of insulin secretion.

Among the Pima Indians themselves, insulin resistance appears to be a major predictor of the subsequent development of NIDDM. Insulin action shows strong familial aggregation in the Pima Indian population. In addition, insulin action in vivo has a trimodal frequency distribution consistent with the hypothesis that a single, co-dominantly inherited, autosomal gene is a major determinant of insulin resistance in the population.

These studies have strongly suggested that the reason for the high prevalence of NIDDM in the Pima Indians is their high prevalence of insulin resistance. Insulin resistance appears to have major genetic determinants and studies are now underway to search for genetic markers for insulin resistance and ultimately, to identify what the genetic abnormality maybe that causes insulin resistance and the high risk of NIDDM.

Mechanisms of insulin resistance. Since insulin resistance appears to be a risk factor for the development of NIDDM, studies have been done to identify the biochemical and molecular mechanisms for this metabolic abnormality. Previous work established that the major tissue involved in insulin resistance is skeletal muscle. We have therefore focused our biochemical studies on the effect of insulin on several skeletal muscle enzymes involved in intermediary metabolism. It is clear that several enzymes are not normally regulated by insulin in insulin resistant individuals. These include glycogen synthase, glycogen synthase phosphatase, phosphorylase phosphatase, protein tyrosine phosphatase, and S6 kinase. However, casein kinase II appears to be normally regulated by insulin in insulin resistant subjects as does the insulin receptor tyrosine kinase activity. In addition to the abnormalities in insulin stimulation, there was also a lower basal activity of glycogen synthase phosphatase and phosphorylase phosphatase in insulin resistant subjects. The basal protein tyrosine phosphatase activity in the particulate fraction of the muscle homogenate of the insulin resistant subjects was higher than that observed among the insulin sensitive subjects. Thus, there are several enzymes with different activities either basally or in response to insulin in the insulin-resistant subjects. If there is a single gene defect causing insulin resistance in the Pimas, we can expect that this abnormality must.be common to each of these pathways.

Studies are currently underway to further define our understanding of the mechanism of the altered basal activity of the glycogen synthase phosphatase as well as the protein tyrosine phosphatase activity (particulate fraction). Attempts are being made to clone and sequence the cDNA of one or more glycogen synthase phosphatases and protein tyrosine phosphatases to identify the specific gene product that is responsible for the altered basal activity and whether the primary base sequence of these genes may be abnormal.

In addition, we have begun studies to look at insulin regulation of gene expression in insulin sensitive and resistant individuals. We have identified several mRNA levels that change in response to insulin infusion in vivo in skeletal muscle, and will soon be assessing whether those mRNA levels respond to insulin similarly in insulin resistant subjects.

Obesity. Obesity is highly prevalent among the Pima Indians and is a major risk factor for the development of NIDDM. Obesity also aggravates insulin resistance. Studies, are therefore being performed to understand how obesity might aggravate insulin resistance and what are the potential etiologic factors causing obesity in the population.

Previous studies have suggested that obesity is associated with a reduced skeletal muscle capillary density. We hypothesized from this observation that there would be lower interstitial insulin levels in obese subjects as compared to lean controls despite the similar insulin levels in the capillaries. However, in vivo studies using cannulation of peripheral lymphatic vessels have failed to confirm this hypothesis. There appears to be similar, and perhaps even higher, insulin levels in the interstitium of obese subjects as compared to lean controls. An alternative hypothesis of how obesity might worsen insulin resistance is that obesity is associated with altered fatty acid composition of plasma membranes which effects insulin signal transduction. We have begun a study to determine the fatty acid composition of plasma membranes of variety of tissues in obese and lean Pima Indians.

Mechanisms of obesity relate to imbalance of energy intake vs. energy expenditure. We have previously observed that individuals vary considerably in the rate of energy expenditure, independent of individual differences in body size and composition, age, and sex. In addition, we have found that a low metabolic rate is predictive of body weight gain. Age does not appear to be a major determinant of the rate of sedentary energy expenditure, but females appear to have lower metabolic rates than males, which may partly explain the greater prevalence of obesity among females. Physical fitness does not appear to effect energy expenditure beyond its effect on body weight and composition.

The mechanism of the low metabolic rate as a risk factor for obesity is under further investigation. Sympathic nervous system activity appears to contribute to maintenance of resting metabolic rate among the Caucasians but this effect is not observed in Pima Indians, suggesting a possible abnormality in SNS - mediated energy expenditure among the Pimas. More detailed studies of the sympathetic nervous system activity is the Pima Indian population are currently underway.

Studies using the doubly-labeled water method have also been undertaken to examine the effect of individual differences in physical activity in free-living conditions on body weight gain. Preliminary studies have validated this method for measuring energy expenditure in humans. The data collected so far show that females have lower physical activity compared to males and that overweight subjects have lower physical activity than lean subjects. Prospective studies need to be completed to prove that this lower physical activity in the obese is a risk factor for subsequent weight gain.

We have observed that a lower rate of lipid oxidation is also a risk factor for subsequent weight gain, independent of the effect of a low metabolic rate. Individual differences in lipid oxidation rates do not appear to be fully explained by different degrees of body composition and we are also investigating the potential biochemical basis for the differences in lipid oxidation rates between individuals.

Awards

Eric Ravussin, Ph.D.

A. Mayer Ward for outstanding contributions in the field of obesity; International Association for the Study of Obesity. Awarded in Kobe, Japan, 1990.

Invited Lectures and Invited Participation in Symposia

Clifton Bogardus, M.D.

"The Role of Insulin Resistance in the Pathogenesis of NIDDM," Endocrine Grand Rounds, University of Pittsburgh, October, 1990.

"The Syndrome of Obesity and Diabetes Mellitus in Pima Indians," Sixth International Congress on Obesity, Satellite Symposium, Diabetes and Obesity, Nagoya, Japan, October, 1990.

"The Pathogenesis of NIDDM and Obesity in Pima Indians," Shiga University, Kyoto, Japan, October, 1990.

"Insulin Resistance and Insulin Secretion in Pima Indians," Sixth International Congress of Obesity, Kobe, Japan, October, 1990.

"Insulin Resistance and Glucose Intolerance," Fifth Scientific Committee Meeting on Diabetes, Bayer Company, November, 1990.

"Pre-Diabetes: Decreased Insulin Sensitivity," Diabetes Symposium, December, 1990.

Keystone Symposia: Diabetes and Insulin Action, January 1991. Chairman of Workshop on Genetics of NIDDM.

"Correlation of Insulin Action in Vivo with Enzyme Modification," Fourteenth International Diabetes Federation Meeting. Symposium on Physiology of Insulin Action in Muscle, June 1991.

"Pathogenesis of NIDDM," Medical Grand Rounds Georgetown University, June 1991.

"Impaired Glucose Tolerance As A Disorder of Insulin Resistance," Nordisk Symposium. Hypertension As An Insulin-Resistant Disorder. Genetic Factors and Cellular Mechanisms, July, 1991.

Eric Ravussin, Ph.D.

"Normalization of Energy Expenditure Results According to Body Weight and Composition," Satellite Conference, Honolulu, Hawaii, 1990.

"Overview of Energy Metabolism, Pharmacological Treatment of Obesity," Satellite Symposium, Yokohoma, Japan, 1990.

"Determinants of Energy Expenditure and Risk Factors for Obesity," Sixth International Congress on Obesity, Kobe, Japan, 1990.

"Determinants of Early Energy Expenditure in Sedentary Humans," British Association for Study of Obesity, London, United Kingdom, 1991.

"Energy Expenditure and Etiology of Type II Diabetes," Symposium "Update on Diabetic Complication", Leuven, Belgium, 1991.

"The Pathogenesis of Obesity in Man," European Course on Clinical Nutrition, Leuven, Belgium, 1991.

"Thermogenesis: A Cause for Weight Gain," Third Annual Conference on Management of the Overweight Patient, Dearborn, Michigan, 1991.

"Daily Energy Expenditure in Man: Measurements in a Respiratory Chamber and by Doubly-Labeled Water," First Clintec International Horizons Conference, Amsterdam, The Netherlands, 1991.

"Genetic Factors in Obesity, Insulin Resistance, and Thermogenesis on Exemplified by the Pima Indian Community," 14th International Diabetes Federation Congres, Washington, DC, 1991.

James Sommercorn, Ph.D.

"Insulin Signal Transduction in Human Muscle," Calbiotechnology, Inc., Seattle, Washington, June, 1991.

Diabetes and Arthritis Epidemiology Section

The Section has continued the longitudinal studies of genetic and environmental risk factors for non-insulin-dependent diabetes and its vascular complications as well as continuing epidemiologic studies of rheumatoid and other forms of arthritis, cholelithiasis, mortality rates and causes of death in the Pima Indians. Cross-sectional studies show strong inverse relationships of habitual physical activity with diabetes and upper body fat distribution, suggesting that physical inactivity contributes to diabetes. The long follow-up provided by the Pima Indian study yields increasingly valuable data on complications of diabetes and the transmission of susceptibility to diabetes and its complications from one generation to the next.

Fasting hyperinsulinemia, presumably reflecting increased insulin resistance, occurs at an earlier age in the offspring of women who had abnormal glucose tolerance during pregnancy, and these offspring are more obese and have higher rates of abnormal glucose tolerance. The mother's 2-hour glucose concentration during pregnancy is also a significant predictor of glucose concentrations during pregnancy in her own offspring. Pima Indian children were found to be more hyperinsulinemic than Caucasian children. These results indicate that insulin resistance, which probably reflects the susceptibility to develop NIDDM, is manifest at a young age as fasting hyperinsulinemia and is affected by the metabolic abnormalities associated with the diabetic pregnancy.

Age-specific mortality rates are high in the Pima Indian population, and frequent causes of death are diabetic nephropathy, ischemic heart disease, and cirrhosis of the liver. Nontraumatic death rates are related to diabetes and inversely related to obesity (body mass index), except for the extremely obese (body mass index at least 40 kg/m squared), in whom death rates were high.

Genetic determinants of diabetes

At least part of the susceptibility to diabetes appears to be transmitted by a major autosomal gene, the location of which is being sought by means of linkage analysis. To this end DNA samples have been collected and EBV-transformed lymphocyte cultures have been established from members of families informative for linkage analysis. Preliminary results using blood typing data routinely collected at biennial examinations indicate linkage of diabetes with the MNSs locus among sibling pairs and an association of diabetes with HLA-A2 type among Pima men.

Diabetes complications are being documented and their risk factors determined. Major complications under study are nephropathy, end stage renal disease, retinopathy, coronary heart disease (CHD), and lower extremity amputation. All of these complications except CHD develop at least as frequently in this population with non-insulin-dependent diabetes as in people with insulin-dependent diabetes.

Diabetic nephropathy

In collaboration with investigators from Stanford University and The Cleveland Clinic, a detailed study of renal function in Pimas with normal or impaired glucose tolerance or diabetes of short or long duration is being performed. These groups are compared with respect to glomerular filtration and pore size distribution, and these functional measures will be related to blood pressure, degree of hyperglycemia, and disease progression during subsequent follow-up. The study indicates a defect in glomerular pore size early in the course of diabetes without a major change in glomerular filtration rate.

Diabetic nephropathy among Pimas with diabetes is highly familial, whether nephropathy is defined as heavy proteinuria or as renal insufficiency. The genetic approaches described above for diabetes will also be applied to studying the genetics of diabetic nephropathy.

Other activities

Section staff continue to be active in medical research and education beyond the projects described here. Staff collaborate extensively in research projects conducted by the Clinical Diabetes and Nutrition Section of the Branch and the National Center for Health Statistics. They continue lecturing at universities and contributing to national and international meetings and workshops. In addition, members of the section lecture regularly at Indian Health Service facilities. Staff have participate in the following national and international meetings:

William C. Knowler, M.D., Dr.P.H.

Visiting Professor, Department of Community Health Sciences, Lund University, Dalby, Sweden (supported by the Swedish Medical Research Council), 1990- .

Faculty, post-graduate course in Molecular Medicine in Diabetes - Etiology, Pathogenesis, and Complications. Department of Medicine, Lund University, Malmö General Hospital, Malmö, Sweden, November 5-9, 1990.

Invited Lectures and Invited Participation in Symposia

William C. Knowler, M.D., Dr.P.H.

"Intervention in IGT and early diabetes in high risk populations on development of diabetes and its complications: existing clinical trials in IGT," National Diabetes Advisory Board, Crystal City, VA, December 10, 1990.

"Pathogenesis and natural history of diabetes and its complications in the Pima Indians," International Diabetes Federation, Washington, D.C., June 26, 1991.

David J. Pettitt, M.D.

"Obesity and diabetes in the offspring of mothers who have developed diabetes during pregnancy: update of the Pima Indian studies," Third International Workshop Conference on Gestational Diabetes Mellitus, Chicago, IL, November 9, 1990.

"Reasons for variation in the prevalence of gestational diabetes," Satellite Symposium to 14th IDF Congress - Diabetes: Effects on Pregnancy and the Neonate, Newport, RI, June 20, 1991.

"Pregnancy and diabetes in Pima Indians," Diabetes, Obesity, and Epidemiology Update Conference for IHS Diabetes Control Officers, Scottsdale, AZ, August 7, 1991.

M. Aline Charles, M.D.

"Natural history of insulin resistance in Pima Indians," INSERM Unit 21, Villejuif, France, December 21, 1990.

"Blood pressure in Pima Indian children," National Conference on Cholesterol and High Blood Pressure Control, Washington, D.C., April 9, 1991.

Biostatistics and Data Management Section

This section is engaged in data management and support activities for the research operations of the Branch as a whole. The major activities are updating, error checking, storage, and retrieval of datasets for the extensive epidemiologic study; providing a computer-based work scheduling system for the radio-immunoassay laboratory; and assistance with many datasets from the studies conducted by the Clinical Diabetes and Nutrition Section. Other major activities include support of laboratory instrument-computer interfacing, adaptation of genetics programs written by non-NIH scientists, and extensive support of microcomputers, including installation of local area networks in Phoenix and Sacaton.

The microcomputer-based system in the outpatient research clinic in Sacaton, Arizona for maintenance and updating of demographic data for the entire study population has been maintained and extended. Work in progress will allow for the scheduling of research procedures in the clinic to be performed by computer.

Consulting on statistical and epidemiologic methods and data management for specific scientific projects has been the other major activity of the Section, on which much of the productivity of the direct research activities of the Branch depends.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69000-26 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Diabetes Mellitus and Other Chronic Diseases in the Gila River Indian Community

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	D.R. McCance	Visiting Associate	DAES, NIDDK
	R. Hanson	Medical Staff Fellow	DAES, NIDDK
	D. Mott	Research Chemist	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service; Ariz. State U.; Cleveland Clinic, Cleveland OH; University of Pittsburgh.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.1	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to identify the determinants of non-insulin-dependent diabetes (NIDDM), various types of arthritis, and gallbladder disease, and elucidate the natural history of the diseases. Genetic and environmental risk factors for NIDDM have been studied in the Pima Indians. The residents of the study area, approximately 5000 people, have participated in a longitudinal population study since 1965, allowing observations of the natural history of diabetes mellitus. Risk factors for obesity, hypertension, and cholelithiasis are also studied, along with the relationships of these diseases to diabetes and their effects on mortality rates. The genetics of diabetes is studied by means of family studies and relationships of genetic markers to disease. The roles of obesity, serum insulin concentrations, impaired glucose tolerance, occupational and leisure-time physical activity and diabetes in relatives are assessed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69001-22 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Complications and Outcome of Diabetic and Prediabetic Pregnancies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service; Karolinska Institut, Stockholm, Sweden (B. Persson) (Foreign); Mayo Clinic, Rochester, Minnesota (B.A. Kottke)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Infants of diabetic mothers are more likely than infants of nondiabetic mothers to exhibit macrosomia, prematurity, perinatal mortality, and congenital malformations. Offspring of diabetic women are also at an increased risk of developing obesity and glucose intolerance during childhood and young adulthood. The purposes of the project are to determine the effects of diabetes and impaired glucose tolerance during pregnancy on outcome of pregnancy in women in the Gila River Indian Community and to determine long-term prognosis for the women and their offspring. By means of a glucose tolerance test and chart review, the diabetes status of every woman is determined during the third trimester of each pregnancy. The characteristics of women who have diabetes or impaired glucose tolerance during the pregnancy are compared with those of women who are normal in pregnancy and subsequently develop diabetes, and with those of women who remain normal. These women and their offspring are followed, and glucose tolerance tests are performed which include measurements of glucose and insulin. Offspring of diabetic women have more diabetes and more obesity than offspring of nondiabetic and prediabetic women. Fasting hyperinsulinemia, presumably reflecting increased insulin resistance, occurrs at an earlier age in the offspring of women who had abnormal glucose tolerance during pregnancy. Thus, the metabolic abnormalities associated with the diabetic pregnancy result in long-term effects on the offspring including insulin resistance, obesity, and diabetes which in turn may contribute to transmission of risk for developing the same problems to the next generation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69006-21 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gila River Indian Community Autopsy and Mortality Study

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.L. Sievers	Guest Researcher	DAES, NIDDK
	Q.Z. Liu	Visiting Fellow	DAES, NIDDK
	L. Striker	Chief	RCBS, NIDDK

COOPERATING UNITS *(if any)*

Pathology Department, Phoenix Indian Medical Center, Indian Health Service, Phoenix, Arizona; Cleveland Clinic Foundation, Phoenix, Arizona;

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.4	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The causes of death and postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects. Medical records are reviewed for the determination of cause of death and for the occurrence of certain serious diseases or complications of diabetes.

The purpose of the study is to relate the outcome and cause of death to events or risk factors measured in life among Pima Indian residents of the Gila River Indian Community, particularly in relation to diabetes, cardiovascular diseases and gallbladder disease. Post-mortem examinations are obtained whenever possible on members of the Gila River Indian Community to ascertain conditions present at the time of death and ascertain cause of death as precisely as possible. In addition, death certificates and all available medical records pertaining to the subjects are obtained and reviewed in a standardized way for evidence of the complications of diabetes, vascular disease, neoplasms and other conditions, which may have been recognized prior to death. The records of the occurrence of such conditions, together with conditions recognized at autopsy, are used to determine the causes of death and incidence of complications associated with diabetes and other conditions identified initially during life by the longitudinal epidemiologic studies in the population.

Morphometric studies of renal glomerular size and composition are being performed to characterize renal changes related to non-insulin-dependent diabetes in the Pima Indian population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 69009-26 PECR

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Natural History of Arthritis and Rheumatism in the Gila River Indian Community

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	S.P. Heyse	Chief	OPECA, NIAMS
	L. Jacobsen	Visiting Associate	OPECA, NIAMS

COOPERATING UNITS *(if any)*
Indian Health Service, Arizona State University; University of Alabama at Birmingham; NIAMS

LAB/BRANCH
Phoenix Epidemiology and Clinical Research Branch

SECTION
Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☒ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The development and progression of osteoarthrosis, rheumatoid arthritis and ankylosing spondylitis are being determined by means of clinical, radiographic and serological examinations carried out prospectively at two-yearly intervals among adults of the Gila River Indian Community (Pima Indians) in Arizona, in conjunction with epidemiological studies of diabetes in the same community. The purpose of this investigation is to ascertain the determinants of these diseases in the population, and to identify factors which predispose to or alter the natural history of progression of the disease. Host factors such as age, sex, and various gene markers including HLA and Gm, together with various potential environmental determinants, such as obesity and evidence of exposure to infectious agents, are being investigated prospectively to determine their relationship to the development of these diseases. Longitudinal data have now been collected for 25 years and represent a unique data set for epidemiological studies of arthritis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69015-09 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cross-Sectional and Longitudinal Study of "Prediabetes" in the Pima Indians

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	B.L. Nyomba	Visiting Associate	CDNS, NIDDK
	M. DeGregorio	Visiting Fellow	CDNS, NIDDK
	R. Ferraro	Staff Fellow	CDNS, NIDDK
	M. Saad	Visiting Associate	DAES, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.4	3.4	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Pima Indians of Arizona have the highest prevalence and incidence rate of non-insulin dependent diabetes mellitus (NIDDM) of any population in the world. We have been studying a subset of this population to determine the metabolic characteristics that predict the development of the disease, and also to determine the exact sequence of metabolic events that occur in individuals that make the transition from normal glucose tolerance to impaired glucose tolerance than on to diabetes. Our observations to date demonstrate that insulin resistance is a very strong predictor of the development of diabetes in the population. The Pimas are more insulin resistant than Caucasian subjects, and this is independent of the effect of obesity to worsen insulin resistance. Also compared to Caucasians, Pimas secrete much more insulin in response to similar degrees of insulin resistance and this is particularly true at early time points after an oral glucose load or after ingesting a standard mixed meal. Insulin resistance in the population shows strong familial aggregation. The frequency distribution of insulin action in the population is distributed trimodally, consistent with a genetic inheritance of an autosomal, co-dominantly inherited gene for insulin resistance. Studies are underway to identify this putative gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69020-07 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Insulin Resistance and the Regulation of Muscle Glycogen Synthase Activity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	B.L. Nyomba	Visiting Associate	CDNS, NIDDK
	H. Mori	Visiting Fellow	CDNS, NIDDK

COOPERATING UNITS *(if any)*

IHS; Div of Biol and Med, Brown Univ., Providence RI (D. Brautigan); Med Coll of Ohio, Dept of Pharm, Toledo OH (Keith K. Schlender); Amylin Corp, San Diego CA (Andrew Young); Dept of Kinesiol, Univ of Illinois, Urbana IL (Abram Katz)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.3	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are currently characterizing the abnormalities for regulation of human muscle glycogen synthesis in insulin-resistant subjects. In insulin-resistant subjects, fasting glycogen synthase phosphatase and phosphorylase phosphatase activities are reduced and fail to show the peak insulin stimulation observed for insulin-sensitive subjects at 10-20 minutes. Using specific inhibitors the abnormal enzyme activities were identified as a type-1 phosphatase (PP-1) in human muscle from insulin-resistant subjects. The reduced PP-1 activity observed in both glycogen and cytosolic subcellular fractions suggested an alteration in the catalytic subunit independent of the glycogen-bound regulatory subunit or G component. The abnormally low fasting PP-1 activity in insulin-resistant subjects persisted following trypsin treatment, suggesting that inhibitors 1 and 2 (characterized regulators of PP-1) are not important determinants of the abnormal phosphatase activity. Western blots indicate an increased concentration of catalytic subunit for PP-1 in the muscle from insulin-resistant subjects. This result suggests that the intrinsic activity or regulation of the catalytic subunit is abnormal in insulin-resistant subjects. The apparent affinity for cAMP activation of muscle cAMP-dependent protein kinase (PKA) is not decreased in insulin-resistant subjects following insulin infusion. In contrast, sensitive subjects show a reduced apparent affinity of their PKA regulatory subunit for cAMP following insulin infusion. This results in inactivation of the kinase and could stimulate glycogen synthase activity. These results suggest that abnormal regulation of both glycogen synthase phosphatase and PKA contribute to the insulin resistance which is secondary to abnormal insulin-stimulated glycogen synthase activity. Glucose metabolism also appears to be regulated in muscle by amylin effects on phosphorylase and abnormal insulin stimulation of glucose 1, 6-bisphosphate concentration in insulin resistant subjects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69021-10 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Energy Expenditure in Pima Indians: Risk Factors for Body Weight Gain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	E. Ravussin	Visiting Scientist	CDNS, NIDDK
	R. Ferraro	Staff Fellow	CDNS, NIDDK
	R. Rising	Staff Fellow	CDNS, NIDDK
	E. Larson	Research Dietitian	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service; Dept. of Medicine, Northwestern Medical School, Chicago, IL, (J. Young); Rockefeller University, New York, NY (R. Leibel)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	2.3	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In our search for the possible causes of obesity in the Pima Indians, we have previously shown using indirect calorimetry (respiratory chamber and venti-lated hoods), that metabolic rate varies between people more than can be explained by individual differences in body size and composition, age and sex. This variability also seems partially genetically determined. More importantly, we have shown that subjects with a low metabolic rate are at greater risk of gaining body weight. We have therefore, investigated the possible causes of the variability of metabolic rate. Our studies have shown that: 1) females have lower metabolic rates than males even after adjusting for differences in body size and composition; 2) age is not a major deter- minant of sedentary energy expenditure in sedentary conditions; 3) physical fitness does not change energy expenditure beyond the difference in body weight and composition; 4) the sympathetic nervous system (SNS) activity is a determinant of metabolic rate in Caucasians, but not in the Pima Indians, suggesting that the decrease in SNS-mediated energy expenditure in Pima Indians may predispose them to obesity; 5) metabolic rate seems to be regu- lated to maintain a genetically determined body temperature. Studies in 10-year old children have shown that Pima Indians are much heavier and fatter than Caucasians, but their metabolic rate is normal for their body size and composition. Studies using the doubly-labeled water method to measure energy expenditure in free-living conditions have shown; 1) the variability in energy expenditure is much larger in free-living conditions than in a respira- tory chamber suggesting large differences in physical activity; 2) females have lower levels of physical activity; 3) overweight subjects have lower physical activity than lean subjects; 4) Intensive training does not result in metabolic adaptations lowering energy requirements in female athletes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69024-05 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

WHO Collaborating Center for Epidemiological and Clinical Investigations in Diabete

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.H. Bennett	Chief	PECR, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK

COOPERATING UNITS *(if any)* World Health Organization, Non-Communicable Diseases Program, Geneva, Switzerland, (Foreign), Other World Health Organization Collaborating Centers for Diabetes (Foreign), China-Japanese Friendship Hospital, Beijing, China (Pan Xiaoren) (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.75	3.60	1.15

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Phoenix Epidemiology and Clinical Research Branch was designated as the WHO Collaborating Center for Design, Methodology and Analysis of Epidemiological and Clinical Investigations in Diabetes in 1986. The purposes of the Center are to collaborate with the World Health Organization in the implementation of the WHO/IDF action program to provide advice, consultation and collaboration with other investigators in the design, methodology and analysis of epidemiology and clinical diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations relating to the etiology and pathogenesis of non-insulin-dependent diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations, and data analysis relating to diabetes and collaborate with those interested in applying such techniques elsewhere. The Center will advise and help in the design of new studies, including on site assistance when necessary. The Center serves to train investigators from many parts of the world in diabetes epidemiology and clinical research.

The Center participates in the WHO Multicenter Study of Vascular Disease in Diabetes, which is examining the mortality and incidence of vascular complications of diabetes among different ethnic groups in different countries.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69025-05 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Treatment of Impaired Glucose Tolerance in Malmöhus County Sweden

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK

COOPERATING UNITS *(if any)*

Lund University, Dalby, Sweden (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The prognosis and effect of treatment of impaired glucose tolerance (IGT) are studied in residents of Malmohus County, Sweden. Mortality rates are determined in over 2000 persons who had glucose tolerance tests in the 1960s, and results are related to glucose tolerance and other factors at baseline. Some of these subjects participated in a randomized treatment study of IGT. New studies in population screening and treatment of IGT are being planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69027-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Insulin Receptor Tyrosine Kinase in Insulin Resistance in Pima Indians

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	B.L. Nyomba	Visiting Associate	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As part of a project investigating the possible role of the insulin receptor tyrosine kinase in the pathogenesis of insulin resistance in Pima Indians, the kinase activity was determined in muscle biopsies obtained after feeding subjects each of a "traditional" high carbohydrate, Pima-type diet, and a "modern", high-fat Western-style diet. The modern diet induced insulin resistance and increased the kinase activity in the majority of subjects. The change in kinase activity were negatively correlated with the changes in insulin action and positively correlated with the changes in plasma insulin levels. When the time-course of the in vivo kinase stimulation was studied, kinase activation was rapid and detectable in less than 10 minutes of insulin infusion. Receptors previously exposed to insulin in vivo displayed a higher kinase activity in vitro, indicating that circulating insulin primes in active receptor molecules for subsequent kinase activation. It is possible that the kinase increases with plasma insulin levels as part of a mechanism compensating for insulin resistance at a distal site. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 69028-03 PECR

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetics of Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	P.H. Bennett	Chief	PECRB, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	S. Lillioja	Visiting Scientist	CNDS, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK

COOPERATING UNITS *(if any)*
Bowman-Gray Medical School, Winston-Salem, NC; Howard Hughes Medical Institute, University of Chicago, Chicago IL; Arizona State University, Tempe, AZ.

LAB/BRANCH
Phoenix Epidemiology and Clinical Research Branch

SECTION
Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION
NIDDK/NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Non-insulin dependent diabetes mellitus is a common chronic disease that develops in most populations in late middle age. The Pima Indians of Arizona have the highest reported prevalence of this disease in the world and in contrast to many populations the disease often presents at an earlier age. As a result of long-term epidemiological studies in the total population, the familial nature of the disease has been well documented, and segregation analyses suggest the possibility of inheritance by a single additive major gene.

This project will search for genetic determinants of NIDDM using the techniques of genetic linkage analysis with restriction fragment length polymorphism (RFLP) and other genetic markers to identify the chromosomal location of inherited determinants of NIDDM in the Pima Indian population. A number of informative pedigrees have been identified and lymphoblast cell lines from informative members of these pedigrees established. DNA from these lymphoblasts is isolated and polymorphic probes applied to search for evidence of linkage of these markers and NIDDM. Probes with established chromosomal locations will be used to screen the genome to detect genetic linkage with NIDDM.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 69029-03 PECR

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)
Regulation of Skeletal Muscle Ribosomal Protein S6 Kinase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name. title. laboratory. and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	M. DeGregorio	Visiting Fellow	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH
Phoenix Epidemiology and Clinical Research Branch

SECTION
Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION
NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS.	PROFESSIONAL	OTHER.
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

We examined the influence of insulin on S6 kinase activity in fibroblasts from skin biopsies of 5 insulin-sensitive and 5 insulin-resistant Pima Indians. Activities of S6 kinase and S6 peptide (RRLSSLRA) kinase were assayed in extracts of cells obtained at 0, 15, 30, 45 and 60 minutes of exposure to 100nM insulin or to carrier. Exposure of fibroblasts, obtained from insulin sensitive subjects, to insulin resulted in a 2.2-fold activation of S6 kinase activity measured in soluble fractions of the cells. Activity was maximally stimulated by 15 min of exposure to insulin and remained elevated through 30 min before declining. In contrast, S6 kinase was activated by only 50% in fibroblasts obtained from insulin-resistant subjects. The timecourse of activation was similar to that seen in cells from sensitive subjects. S6 peptide kinase activity was stimulated 35% over basal activity in response to insulin in cells obtained from both sensitive and resistant subjects. The activation was maximal by 15 min and declined to basal levels thereafter. These results support a genetic basis of abnormal insulin signal transduction in Pima Indians and suggest that the gene or genes responsible for insulin resistance encode a protein(s) that contributes to the mechanism of insulin action intracellularly. Because activation of S6 peptide kinase was normal in cells from resistant subjects, it is likely that the genetic lesion causing impaired insulin signal transduction may affect only some aspects of insulin action.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69030-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Contribution of Protein Tyrosine Phosphatase to Insulin Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS/NIDDK
Others:	C. Bogardus	Chief	CDNS/NIDDK
	M. Kunkel	IRTA	CDNS/NIDDK
	S. Norman	IRTA	CDNS/NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Cold Springs Harbor Labs., CSH, NY (N. Tonks)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, AZ, 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We previously found that insulin rapidly suppresses the activity of a soluble PTPase in skeletal muscle of insulin-sensitive subjects, but not in subjects resistant to insulin and that activity of a particulate PTPase is chronically elevated in muscle of resistant subjects. Because either of these abnormalities could be responsible for insulin resistance, we have begun to identify and characterize the PTPases in human skeletal muscle. Putative PTPase cDNA sequences were amplified by PCR from total RNA isolated from human muscle using Taq polymerase and degenerate oligonucleotide primers based on conserved amino acid sequences in known PTPases. PCR products of the expected size (340 bp) were purified by agarose gel electrophoresis and reamplified. The second round PCR product was purified and cloned. Analysis of 50 clones by either direct sequencing or by restriction enzyme analysis revealed 12 different PTPase-like sequences. Two of the sequences are unique and are represented by multiple clones. Two others are identical to previously described PTPases (PTP1B and TCPTP) and one is similar to a third PTPase (PTPH22) except for a single amino acid substitution. The amplified sequences occur at frequencies ranging from 2% to 38%. Five other clones differ from TCPTP by only one base. The 340 bp PCR product was used to probe a Northern blot of total RNA from human skeletal muscle. This revealed two mRNA bands of about 4.7 kb and 2.4 kb. Initial screening of a human skeletal muscle cDNA library with the 2 novel PCR products has identified several positive clones for each.

PHS 6040 (Rev. 1/84)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69031-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Phosphorylase Phosphatase by Insulin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	S. Norman	IRTA	CDNS, NIDDK
	B. Thompson	IRTA	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Impaired activation of glycogen synthase in insulin-resistant subjects is associated with both chronically lower activity of protein phosphatase 1 (PP1) and a smaller magnitude of activation of PP1 in response to insulin compared to that seen in insulin-sensitive subjects. Although PP1 activity is low in muscle of resistant subjects, the concentration of catalytic subunit of PP1 (PP1-C), as determined by Western blotting, is higher than in sensitive subjects (B. Nyomba and D. Mott, CDNS, PECRB). Because both of these abnormal characteristics of PP1 in insulin resistance could result from a mutation in the gene for PP1-C, we have begun to examine PP1-C cDNA sequences in muscle of sensitive and resistant subjects and to test the influence of insulin on the concentration of PP1-C RNA in muscle. PP1-C cDNA was amplified by polymerase chain reaction (PCR), ligated with an appropriate vector, cloned and sequenced. Five PCR clones from muscle of an insulin-sensitive subject are virtually identical to the human liver PP1-C cDNA sequence from +138 to +558, except for a base pair change (A→C) at nucleotide +237. Several of these clones have an additional base pair change, but since these occur randomly throughout the sequence, they probably arise from the infidelity of Taq polymerase. Three PCR clones of PP1-C from muscle of an insulin-resistant subject have also been sequenced. Two clones are identical to the sequence obtained from muscle of an insulin-sensitive subject. The third clone differs by a 2bp deletion. The concentration of PP1-C RNA was assayed using a 32p-labeled oligonucleotides probe in an S1-nuclease protection assay. The concentration of RNA corresponding to PP1-C in human skeletal muscle decreased by 50% after 30 min of a high dose infusion of insulin in vivo. The concentration returned to basal levels by 120 min.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69032-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Skeletal Muscle Casein Kinase II by Insulin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)*

PI:	J. Sommercorn	Senior Staff Fellow	CDNS/NIDDK
Others:	C. Bogardus	Chief	CDNS/NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, AZ, 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have investigated the influence of insulin on the activity of casein kinase II (CKII) in skeletal muscle of 7 insulin-sensitive, 4 insulin-resistant, non-diabetic, and 5 diabetic Pima Indians during a 2-hour hyperinsulinemic, euglycemic clamp. In sensitive subjects, CKII was transiently activated (42%) reaching a maximum over basal activity at 45 minutes before declining. CKII was also stimulated in resistant subjects (19%) and diabetics (34%), but the activity remained elevated throughout the clamp. The magnitude of activation of CKII by insulin in muscle of resistant subjects may have been limited by the fact that basal CKII activity in this group was 40% higher than in either sensitive or diabetic subjects. The higher basal CKII activity in resistant subjects was not explained by a higher concentration of CKII protein as determined by Western blotting. Among the three groups, basal CKII activity was correlated with fasting plasma insulin concentrations suggesting that the higher basal activity in resistant subjects resulted from their higher plasma insulin concentrations. Extracts of muscle obtained from all three groups either before or after insulin administration were treated with immobilized alkaline phosphatase, which reduced and equalized CKII activity in the extracts. These results suggest that insulin stimulates CKII activity in human skeletal muscle by a mechanism involving phosphorylation of either CKII or of an effector molecule and support the idea that elevated basal activity in resistant subjects results from insulin action. Our studies suggest that the ability of insulin to activate CKII in skeletal muscle is not impaired in insulin-resistant Pima Indians, and that the biochemical lesion responsible for insulin resistance occurs either downstream from CKII or in a different pathway of insulin action. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69033-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Relationship between insulin resistance and blood pressure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK
	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	E. Ravussin	Visiting Scientist	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epidemiologic evidence has been collected in several population groups in the world demonstrating significant associations between hyperinsulinemia and hypertension. Patients with essential hypertension have been demonstrated to be insulin resistant by direct measurements by several investigators. It has been postulated, therefore that insulin plays a role in the pathogenesis of hypertension through a stimulation of the sympathetic nervous system, by promoting renal sodium retention, or by affecting cation transport. However, in the epidemiologic survey of Pima Indians no relationship between hypertension and serum insulin concentrations were found. To explore this further, the relationship between insulin resistance and blood pressure was studied in Pima Indians, Caucasians and Blacks. Insulin resistance is correlated with blood pressure in Caucasians but not in Pimas or Blacks. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69034-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The long Q-T interval syndrome in diabetes mellitus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Diabetic autonomic neuropathy is frequently associated with a long Q-T interval on electrocardiogram. Long Q-T interval is believed to increase the susceptibility of these patients to ventricular arrhythmias and sudden death. 50 diabetic subjects with a long Q-T interval, 50 diabetics with normal Q-T interval, 50 nondiabetics with a normal electrocardiogram were studied in detail. All underwent detailed evaluation of the autonomic nervous system as well as ambulatory cardiac monitoring to determine the frequency of silent ischemic episodes and ventricular premature beats. A long Q-T interval was found to be equally prevalent in diabetics with or without evidence of autonomic neuropathy. Neither group of diabetics had significant frequency of ventricular premature beats. This study has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69035-03 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Autonomic nervous system activity in obesity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK
	E. Ravussin	Visiting Scientist	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Pima Indians have an extremely high prevalence of obesity, with more than 70% of the adult population having a body mass index of over 27 kg/meters squared. Many animal models of spontaneous obesity, as well as obesity secondary to lesions of one hypothalamus, have been associated with abnormal sympathetic nervous system activity. Recent studies in obese Caucasians demonstrated an apparent abnormality of the sympathetic nervous system activity in obese subjects. This study was designed to study the relationship between obesity and autonomic nervous system activity in the Pima Indians as compared to an age, weight matched group of Caucasians. Preliminary analyses suggest that SNS activity contributes to stimulation of the metabolic rate in Caucasians but that this stimulation is absent in Pimas. This study has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69036-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Epidemiology of Complications of Non-Insulin-Dependent Diabetes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.A. Charles	Special Volunteer	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	D.R. McCance	Visiting Associate	DAES, NIDDK
	R. Hanson	Medical Staff Fellow	DAES, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service; State University of New York at Buffalo; Cleveland Clinic Foundation, Cleveland, OH.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	2.0	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of the project is to determine the incidence rates, rates of progression, and risk factors for the chronic complications of NIDDM. The study is conducted in the Pima Indians of the Gila River Indian Community, who have participated in a longitudinal epidemiologic study since 1965 (see project Z01 DK 69000).

Risk factors for the major complications of diabetes, retinopathy, nephropathy, coronary artery disease, and peripheral vascular disease are determined by longitudinal followup of diabetic subjects. Methods of ascertainment of these complications include fundus photography, measurement of urine albumin and serum creatinine concentrations, electrocardiography, and documentation of lower extremity amputations.

These complications are responsible for the major morbidity and excessive mortality associated with diabetes in this population. The incidence rates of severe complications such as end stage renal disease, cataract requiring surgical treatment, and lower extremity amputations are as high among diabetic Pimas as reported anywhere else in the world. Diabetic nephropathy is the leading cause of deaths among diabetic Pimas. Late stages of nephropathy can be predicted by minor abnormalities of urinary albumin excretion early in the course of the disease and show strong familial aggregation suggesting a genetic susceptibility to nephropathy as well as to diabetes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69037-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Kidney Function in Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK

COOPERATING UNITS *(if any)*

Cleveland Clinic Foundation; Stanford University; Emory University; Chronic Renal Diseases Program, NIDDK

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The functional characteristics of the renal glomerulus are being investigated in Pima Indians of the Gila River Indian Community to identify the underlying pathogenetic mechanisms involved in the initiation and progression of renal disease in non-insulin-dependent diabetes mellitus (NIDDM).

The Pima Indian population has a high incidence of NIDDM and diabetic nephropathy. Six groups of subjects are being studied: subjects with normal glucose tolerance, individuals with impaired glucose tolerance (IGT), those with newly diagnosed diabetes (<3 years duration NIDDM), and diabetic subjects ($\geq$5 years duration NIDDM) with evidence of (a.) mild abnormal albumin excretion (b.) severe abnormalities of albumin excretion (c.) normal albumin excretion. Measurements of renal and glomerular capillary wall function including glomerular filtration rate (GFR), renal plasma flow (RPF), dextran sieving coefficients, and albuminuria are being performed and correlated. Markers and/or predictors of progression as well as the mechanisms of initiation and progression of diabetic renal disease are being sought.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69038-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Insulin and Hypertension in Pima Indians

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	Q.Z. Liu	Visiting Fellow	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	P.H. Bennett	Chief	PECRB, NIDDK

COOPERATING UNITS *(if any)*

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It has been suggested that subjects with diabetes or impaired glucose tolerance (IGT) have a higher prevalence of hypertension than subjects with normal glucose tolerance (NGT). Both obesity and glucose intolerance are related to increased insulin resistance, hyperinsulinemia, and increased risk of hypertension. Moreover, patients with essential hypertension are insulin resistant and hyperinsulinemic. Thus, it has been postulated that insulin might be involved in the pathogenesis of hypertension. The incidence of hypertension by glucose tolerance was determined in 2784 Pima Indians without clinical proteinuria at baseline, and the associations between insulin treatment or endogenous insulin concentration and incidence of hypertension were examined among these subjects.

Subjects with diabetes or IGT had a higher incidence of hypertension than did subjects with NGT. Among all diabetic subjects, exogenous insulin (insulin treatment) predicted subsequent hypertension. Among nondiabetic subjects, but not diabetic subjects treated with insulin, endogenous fasting insulin concentration predicted subsequent hypertension. Thus, these findings support the hypothesis that insulin plays a role in developing hypertension in non-diabetic Pima Indians.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69039-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Dietary Survey of the Pima Indians of the Gila River Indian Community

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK

COOPERATING UNITS *(if any)*

Cleveland Clinic Foundation, Phoenix, Arizona.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

  ☐ (a1) Minors

  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An age-sex-stratified sample of 600 residents of the Gila River Indian Community, ages 18-75 years, was recruited for a dietary survey. Dietary intake was estimated by the dietary history method to obtain quantitative food frequency information. Reported energy intake was higher in men and negatively related to age; thus, relationships with weight were analyzed by multiple regression controlling for sex and age. Energy intake in kilocalories (kcal) was positively associated with body weight or body mass index, adjusted for age and sex. Body weight was associated with absolute intake of the major dietary components, carbohydrate, fat, and protein, but not with any of these components expressed per 1000 kcal, suggesting that total energy intake, rather than proportions of specific components, was the variable having the strongest association with body weight. Alcohol consumption (in any amount) was higher in men and inversely associated with age and weight. Neither energy intake nor specific components were significantly associated with diabetes, after adjustment for age and sex, nor did diabetes affect the relationship between energy and weight, in a subset of 307 subjects (153 diabetics) examined for diabetes within one year of the diet history. The acceptance of obesity in this population may reduce the under-reporting of energy intake which has been postulated in other studies. Body weight in Pima Indians is weakly but positively associated with increased caloric intake.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69040-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Sodium-Lithium Countertransport and Blood Pressure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK

COOPERATING UNITS *(if any)*

University of Pittsburgh.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Red blood cell sodium lithium countertransport is a genetic marker of hypertension in several ethnic groups. It reflects the sodium-hydrogen antiport activity in renal tubules. Several recent studies showed an association between sodium-lithium countertransport and predisposition to diabetic nephropathy. We are studying the relationships between sodium-lithium countertransport, nephropathy, and blood pressure in a sample of 200 Pima Indians.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69041-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Insulin Resistance in Obesity and the Association with Lymph Insulin Kinetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service (C. Castillo), University of Southern California (R. Bergman)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously shown that the density of capillary supply in skeletal muscle of man correlates with insulin resistance. We postulated that since the unfenestrated capillaries of muscle are relatively impermeable to insulin then the increased insulin resistance in those with low capillary density might be due to altered kinetics of insulin penetration to its sites of action in muscle. We have published a method of directly collecting lymph from a peripheral lymphatic vessel in sufficient amounts to measure insulin, and glucose during changes in arterial insulin. Insulin concentrations in limb lymph are much lower than in plasma and incontrast to plasma are highly correlated with glucose uptake in each individual. However, compared to obese insulin resistant subjects, the slope of the glucose uptake/insulin relationship is much steeper in lean subjects. Hence, interstitial insulin concentrations determine insulin action but individual variations is insulin resistance are determined more distally in the pathway leading to insulin mediated glucose uptake.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69042-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of nicotinic acid-induced insulin resistance on beta-cell function.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M.F. Saad	Visiting Associate	DAES, NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Individuals with non-insulin dependent diabetes mellitus have both insulin resistance and abnormal islet cell function in response to glucose. Which of these lesions is a primary defect remains controversial. One way to address this question is by studying the effect of insulin resistance on beta-cell function in individuals with normal islet cell function, and with no family history of diabetes, to determine if islet function "normally" deteriorates in response to insulin resistance. In this study, insulin resistance was induced by administration of nicotinic acid in young, lean, healthy Caucasian males with no family history of diabetes. Nicotinic acid induced severe degrees of insulin resistance and marked deterioration in glucose tolerance. Insulin secretion increased in response to the insulin resistance but was not sufficient to overcome the insulin resistance, thereby, resulting in the deterioration of glucose tolerance. These data suggest that a "normal" pancreas does not fully compensate for insulin resistance. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69043-02 PECR

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of gene expression by insulin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Sommercorn	Senior Staff Fellow	CDNS/NIDDK
Others:	C. Bogardus	Chief	CDNS/NIDDK
	B. Thompson	IRTA	CDNS/NIDDK

COOPERATING UNITS *(if any)*

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, AZ, 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Insulin resistance in Pima Indians appears to result from a postreceptor defect in signal transduction that affects the ability of insulin to activate glycogen synthase in skeletal muscle. Because the mechanism by which insulin affects nuclear events may differ from that by which it alters metabolic processes, we examined the influence of insulin on concentrations of RNA corresponding to multiple loci in human muscle in vivo. Multiple RNA species were assayed simultaneously in a single sample of total RNA by S1 nuclease protection using specific oligonucleotides that can be resolved on a DNA sequencing gel, excised and quantitated by liquid scintillation spectrometry. RNA levels determined by the multiple S1 nuclease method are comparable to estimates obtained by Northern analysis. Levels of RNA corresponding to Glut-4, Glut-3 and the insulin receptor were unchanged over the two-hour time course of insulin administration. RNAs for c-fos and c-jun were increased two-fold, but the changes were not statistically significant. RNA corresponding to c-src, c-Ha-ras, c-myc, and myf-5 all displayed 2-3 fold transitory increases that were statistically significant. In contrast, RNA corresponding to type 1 protein phosphatase catalytic subunit decreased 50% by 30 minutes of insulin infusion and returned to basal levels by 120 minutes. We will determine next if insulin resistance impairs the ability of the hormone to alter expression of these loci.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-69044-01-PECRB

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Insulin resistance in obesity and the associations with membrane phospholipid concentrations.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK

COOPERATING UNITS *(if any)*
Indian Health Service; Garvan Institute Sydney Australia (L. Storlein)

LAB/BRANCH
Phoenix Epidemiology and Clinical Research Branch

SECTION
Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION
NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
  ☐ (a1) Minors
  ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A convincing mechanism for the development of insulin resistance with the development of obesity has not yet been determined. A recent report has indicated that fasting plasma insulin concentration,a measure of insulin resistance is correlated with muscle phospholipid polyunsaturated fatty acid content. A similar relationship is also found with obesity. In a collaborative study with the authors of this work, we will examine the relationship of obesity, insulin resistance and membrane phospholipid content in Pima Indians to determine if obesity leads to insulin resistance by altering membrane phospholipid content.

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